Contrast-enhanced sonothrombolysis for the acute ischemic limb

Nederhoed, Johanna Hilda

2021

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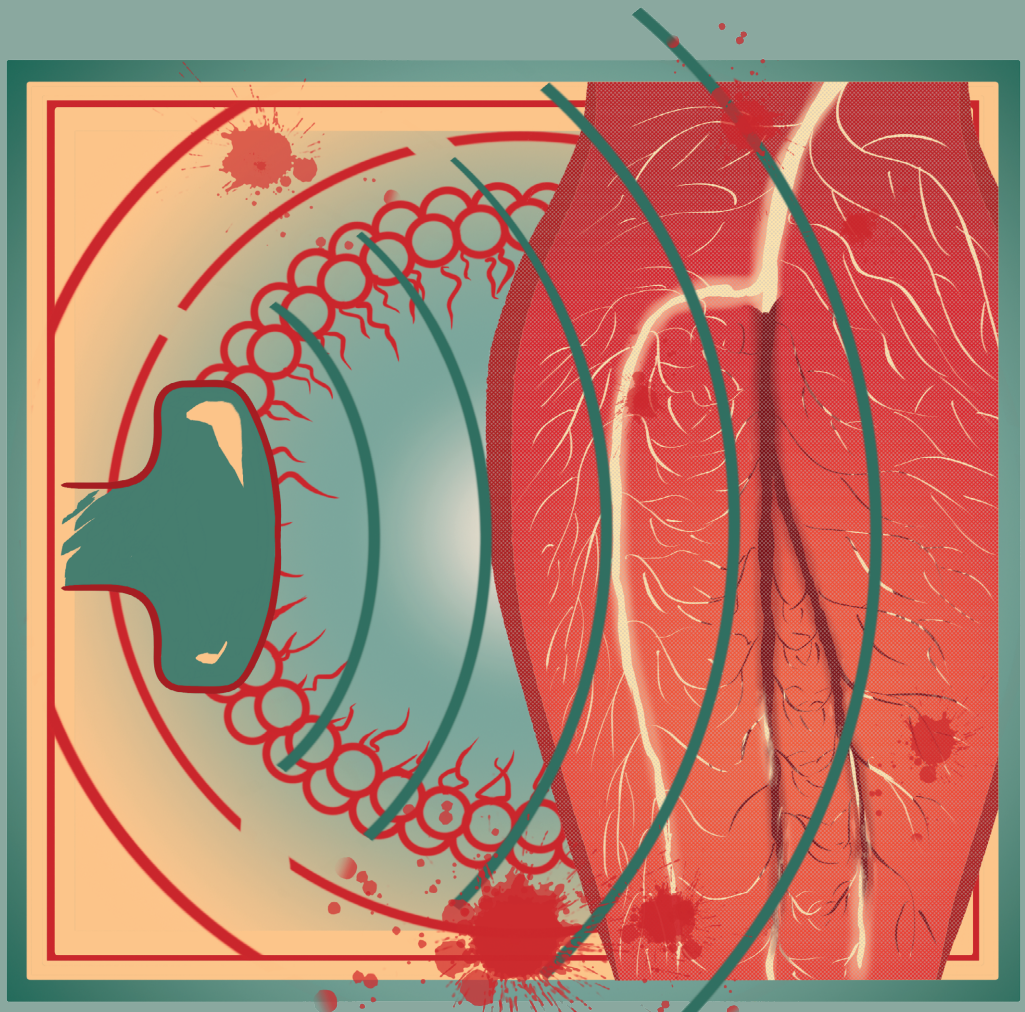

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Contrast-enhanced sonothrombolysis for the acute ischemic limb

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in het openbaar te verdedigen
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van de Faculteit der Geneeskunde
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door

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geboren te Oron, Nigeria

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Euverkant van de nach

Ik kôm neet nao hoés
Vannach gaon ik door
De stad is mien thoés
En haos idder oor

Zeen ik weer waat niejs
En huur ik ein leed
Zô'n prachtige wiés
Die kôs ik nog neet

Die dreej daag in 't jaor
Gen slaop, gen tiéd, noëit klaor
Kôm mei nao d'n euverkant van de nach
We gaon!
..

Frans Pollux, 2013

Contents

Chapter 1: Introduction and outline of the thesis

The acute ischemic limb

Limb ischemia occurs when blood flow to a limb is not sufficient to meet its demand, for instance when a thrombus occurs in an artery. The thrombotic occlusion of the artery leads to insufficient blood flow, causing hypoxia of the tissue and in a later stage depletion of nutrients necessary to maintain cellular function.

Limb ischemia may be acute or chronic, depending on duration of symptoms before presentation. We talk about chronic limb ischemia when symptoms exist for over 2 week and we classify this according to Fontaine into 4 categories: the mildest being asymptomatic and the most severe consisting of loss of tissue (table 1).

Table 1. Classification of ischemia according to Fontaine[1]

Stages	Symptomatology
Stage I	Asymptomatic
Stage IIa	Intermittent claudication > 200m
Stage IIb	Intermittent claudication < 200m
Stage III	Ischemic rest pain
Stage IV	Necrosis / gangrene

Limb ischemia is considered acute when symptoms exist for less than 2 weeks. In case of acute limb ischemia, tissue loss might not be visible yet. Severe pain in rest and loss of function are the most frequently presenting symptoms. Other symptoms might include discoloration and / or a lower temperature when compared to the contralateral side (table 2). The yearly incidence of acute lower limb ischemia varies between 9 and 26 per 100.000 persons[3-5]. Upper limb ischemia accounts for another 1.2-3.5 cases per 100.000 persons[6,7].

Although limb ischemia is caused by insufficient blood flow, the severity of it is strongly influenced by the duration of symptoms. In ischemic conditions, cells will switch to an anaerobic metabolism. Cellular pH will decrease due to production of lactate. At the same time, a decrease in ATP will cause a dysfunction in ATPase-dependent ion transport mechanisms. Fluid will enter the cell, leading to edema and eventually rupture of the cell membrane. Another effect of ischemia is an increase in intracellular calcium and with it the production of proteases that can further damage tissue. The longer ischemia persists, the more tissue will be lost, and the more byproducts of ischemic metabolism will be present. After restoration of blood flow, reperfusion will initially exacerbate injury to the tissue. This is called the ischemia-reperfusion injury. Once blood flow to the limb is restored, reactive oxygen species (ROS) will be generated and a cascade of (systemic) inflammatory and thrombogenic processes will be triggered. Locally, these processes will cause

further disruption of the cellular structure and cell death. Endothelial dysfunction will cause more leakage of fluid leading to edema and reactive vasoconstriction[8].

Table 2. Classification of acute ischemia according to Rutherford[2]

Category	Prognosis	Physical findings		Doppler	
		Sensory loss	Motor deficit	Arterial	Venous
I Viable	Limb not immediately threatened	None	None	Audible	Audible
II Threatened					
a. Marginally	Salvageable if promptly treated	Minimal (toes) or none	None	Inaudible	Audible
b. Immediately	Salvageable with immediate revascularization	More than toes, rest pain	Mild, moderate	Inaudible	Audible
III Irreversible	Major tissue loss or permanent nerve damage inevitable	Profound, anesthetic	Profound, paralysis	Inaudible	Inaudible

Acute peripheral arterial occlusion leads to loss of the effected limb in 10-30% of cases[9]. And not just limb-threatening, ischemia is also life threatening. Thirty-day mortality in acute lower limb ischemia is reported to be between 15 and 20%[10]. Detrimental factors are both the ischemia and the reperfusion, but also complications of treatment and time to intervention. In a study by Kendrick et al, mortality rates increased from 15 to 48% when surgical treatment was delayed 6 hours[11].

Current standard of care

There is a trend in etiology of acute limb ischemia. Where earlier reports, from the 1980s, show an embolic origin of the arterial occlusion in up to 81% of cases, this pathology has decreased in later studies to 14%[12-14]. In the remaining 86% of cases, acute limb ischemia is caused by thrombosis occurring in arteries with pre-existing peripheral arterial disease, in bypasses or in a peripheral arterial aneurysm. With the changing etiology of acute limb ischemia and advancing medical technology, comes a paradigm shift in standard of care. More and more, traditional surgical treatment is reserved for patients with an acute embolic event, whereas less invasive endovascular interventional techniques are used for acute arterial thrombosis. A recent study by Davis et al in patients with Rutherford grade

IIa and b, showed that only 13% of patients had received traditional open surgical revascularization[15]. In comparison, a study by Eliason et al in treatment of acute lower extremity ischemia between 1992 and 2000, showed 47.3% of patients received surgical embolectomy[16].

The most frequently used endovascular intervention for acute peripheral arterial thrombosis is intra-arterial catheter directed thrombolysis. First introduced by Verstraete et al in 1963, catheter directed intra-arterial thrombolysis became part of standard care during the 1990s[17]. A catheter is placed at -or through- the site of the arterial occlusion and fibrinolytic medication is continuously infused locally in order to dissolve the thrombus (fig. 1). Repeated angiography shows the status of the lysis and possible underlying pathology. This therapy has the benefit of being minimally invasive with less or no surgical trauma. The fibrinolytic medication can reach even the smallest vessels, minimizing loss of outflow. There is no need for general anesthesia and once the clot has dissolved, angiography shows the underlying pathology which can then be addressed in the same session.

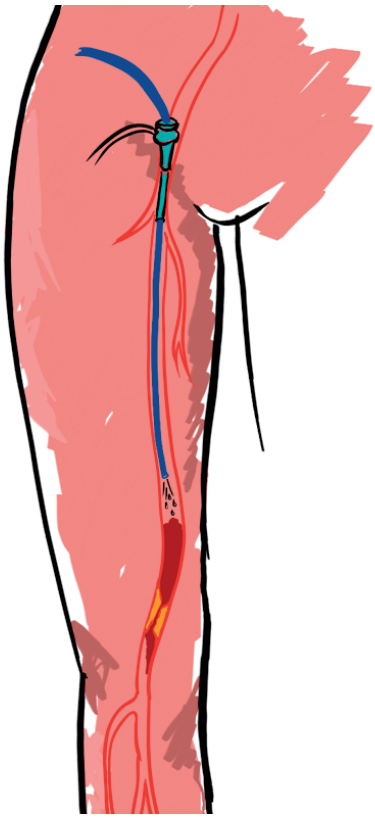


Figure 1. Intra-arterial catheter-directed thrombolysis.

The skin is punctured and a sheath (teal) is placed in the right common femoral artery. The intra-arterial catheter (dark blue) guides the infusion of the fibrinolytic agent to the site of the occlusion. After the clot (dark red) has dissolved, the underlying atherosclerotic lesion (yellow) can be addressed.

Although thrombolytic therapy is minimally invasive, it still requires puncture of the skin and artery for placement of the intra-arterial catheter. Furthermore, it takes time to dissolve a clot using a fibrinolytic agent, while prognosis is strongly related to time. During treatment, patients must adhere to a hospital bed, adding to the burden for the patient. The therapeutic aspect of fibrinolytic agents, to dissolve clots, also carries risks. Major bleedings occur in 8,9% of patients and the most feared complication, intracranial hemorrhage, occurs in 1-2.8% of patients[18,19]. There is a strong correlation between the risk of complications of thrombolysis and the duration of infusion. In a summary of evidence by van den Berg, the risk of complications was 4% if fibrinolytic therapy had a maximum duration of 8 hours. This increased to 34% at a duration of 40 hours[19].

Possibilities for improvement of current standard of care

Minimal invasive techniques for revascularization in acute peripheral arterial occlusion have clear benefits for the patients, however, there is still ample room for improvement. A better prognosis for both limb and life can be expected, should we be able to decrease time to reperfusion of the limb. The burden of treatment for patients with acute limb ischemia is another factor that might be improved. If we can negate the need for an intra-arterial catheter, patients would not have to be bedridden during treatment and skin puncture would only be necessary for placement of an intravenous catheter. Reducing risks of fibrinolytic therapy, especially risk of hemorrhage, is another possible improvement.

In 1995, Tachibana et al introduced the use of ultrasound contrast agents and ultrasound for thrombolysis[20]. They found that the presence of ultrasound contrast agents induced acceleration of thrombolysis by ultrasound in an *in-vitro* setting. Ultrasound contrast agents consist of gas-filled microbubbles. They are approximately 1-5μm in diameter, slightly smaller than an erythrocyte (6-8μm), and can freely pass the capillary bed. Under influence of ultrasound, microbubbles start to oscillate (fig. 2). When the ultrasound pressure gets high enough, the microbubbles will destabilize and eventually burst, causing microstreams in the surrounding fluid. When oscillation and/or implosion of microbubbles is induced in the vicinity of a clot, this leads to shear stress to the surface of the clot (fig. 3). Not only does this directly lead to erosion, but the larger surface area of the clot makes it more susceptible to a fibrinolytic agent[21,22].

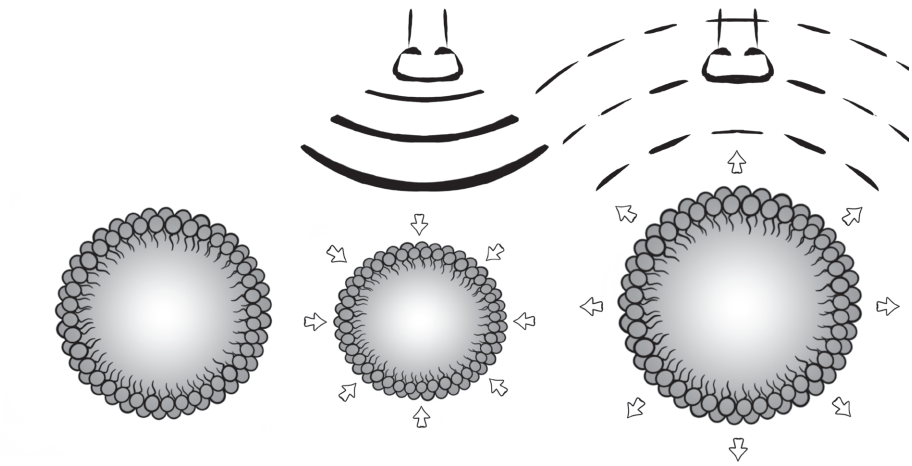


Figure 2. Under influence of positive and negative pressure waves induced by the ultrasound beam, the microbubble will start to oscillate: it will be alternatingly compressed and stretched. Higher pressure waves will lead to collapse of the microbubble.

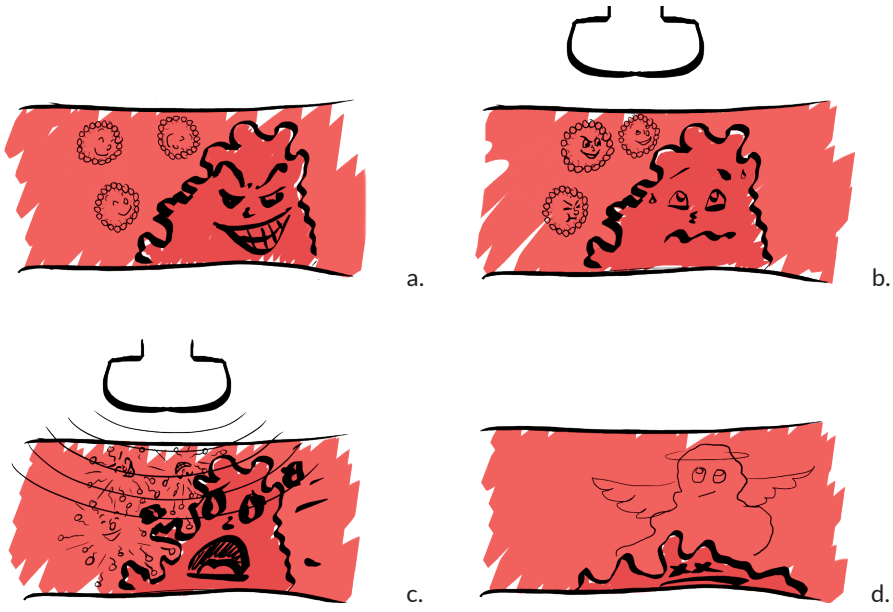
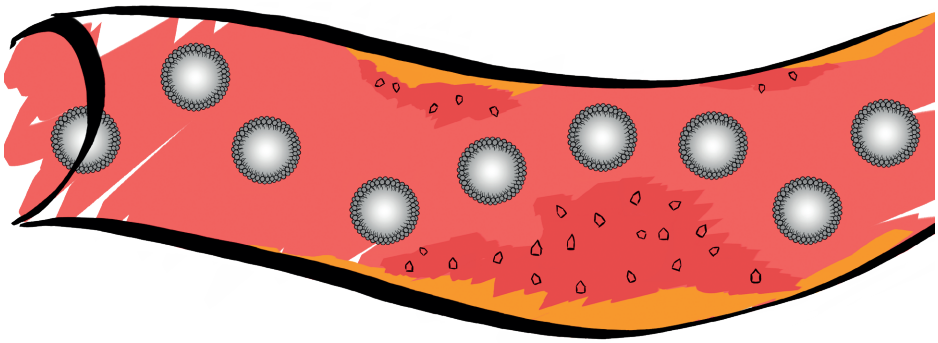


Figure 3. Mechanics of thrombolysis using microbubbles and US.
a. Thrombus has lodged itself in an artery and microbubbles have been administered; b. An US probe is directed at the site of the thrombus; c. High mechanical index US causes the microbubbles to burst, leading to erosion of the thrombus surface; d. The thrombus has dissolved and blood flow has been restored.

So microbubbles can accelerate thrombolysis when influenced by ultrasound. But microbubbles have other interesting properties that could help improve on standard catheter directed thrombolysis. They can be used as a vehicle to transport drugs such as a fibrinolytic agent. This can be achieved by loading drugs into the microbubble or attaching them to its surface membrane. The possibility to attach a drug to the surface of the microbubble also gives us the option to give microbubbles a target to adhere to. We can target microbubbles to adhere to thrombus by attaching Arg-Gly-Asp-Ser, the recognition and binding site of platelet membrane glycoprotein 2b/3a receptor, to its outer layer[23](fig.4). And in recent years, it has even become possible to direct microbubbles magnetically to the site of interest[24].

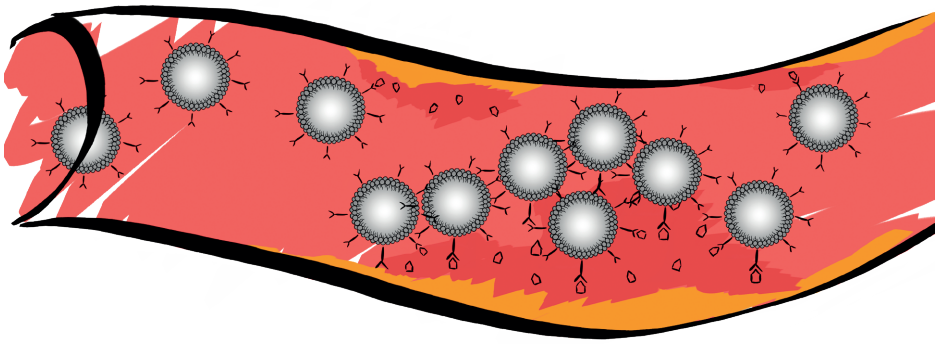
Figure 4. Non-targeted vs targeted microbubbles

a.



Microbubbles follow the blood flow and can be destroyed once they reach the area of the clot. If they are not destroyed, they will continue following the flow.

b.



After following the blood flow, microbubbles that are targeted to adhere to the platelet membrane glycoprotein 2b/3a receptor will cluster at the site of the clot.

Rationale and aims of this thesis

Thrombolysis using a combination of microbubbles, ultrasound and a fibrinolytic agent is called contrast-enhanced sonothrombolysis (CEST). Several studies investigated the benefit of CEST in a setting of acute ischemic stroke or myocardial infarction, but precious little has been published on the use of CEST for peripheral arterial thrombosis. This thesis focuses on different aspects of CEST that might improve treatment for patients with an acute ischemic limb. To give an idea of the current situation and gain some insight into the extent of the problem, we retrospectively reviewed our clinical results with standard intra-arterial catheter-directed thrombolysis and gave an overview of the current status of CEST in acute peripheral arterial thrombosis in a systematic review. We investigated different aspects of microbubbles and different protocols for CEST in a laboratory setting: Firstly, we added microbubbles and ultrasound to standard catheter directed thrombolysis, as this might accelerate thrombolysis. We continued our experiments using microbubbles as a carrier for a fibrinolytic agent, as this might one day negate the need for an intra-arterial catheter. And thirdly, we investigated CEST using the EkoSonic™ Endovascular System (EKOS; Boston Scientific, Marlborough, USA). This system uses an intra-arterial catheter that helps to physically disrupt thrombus by applying local ultrasound. We tested what effect this catheter might have on intra-arterially infused microbubbles and whether the combination of the two would improve thrombolysis without a need for external ultrasound. The findings of these preclinical trials were translated to a safety and feasibility study on the use of microbubbles and ultrasound in standard catheter-directed thrombolysis in a clinical setting.

Outline of the thesis

Chapter 1 provides an insight into the clinical problem and the issues accounted when treating the condition of acute limb ischemia.

The first part of this thesis focuses on the current situation and what is known about CEST in peripheral arterial occlusion, In *chapter 2*, we investigate protocols currently in use for fibrinolytic therapy and explore their risks and benefits. A systematic review of the current literature on therapeutic use of microbubbles in acute peripheral arterial thrombosis is given in *chapter 3*.

The second part of this thesis gives the results of our pre-clinical studies.

In *chapter 4* we present an animal model for investigating different fibrinolytic therapies. This model is implemented in *chapters 5, 6 and 7*, where we explore the therapeutic use of ultrasound contrast agents in fibrinolytic therapy and the possible benefits that can be gained when adding these agents to the current standard of care.

The third part of this thesis focuses on CEST in a clinical setting. In *chapter 8*, chapters 5, 6 and 7 are translated to a clinical study, where we propose a model for microbubble and ultrasound enhanced thrombolysis in setting of peripheral arterial thrombosis. In *chapter 9* we present the results of this clinical trial.

Chapter 10 summarizes the thesis, provides a general discussion, and reflects on the findings in respect to clinical practice as well as gives suggestions for further investigations.

Chapter 11 is a Dutch summary of the thesis.

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’n Beetje

Sterven doe je niet ineens,
maar af en toe een beetje
en alle beetjes die je stierf,
’t is vreemd, maar die vergeet je,

het is je dikwijls zelfs ontgaan,
je zegt ik ben wat moe,
maar op een keer dan ben je aan
je laatste beetje toe.

Toon Hermans, 1983

Part I

Current situation and overview of the literature

Chapter 2. Low-dose thrombolysis for thromboembolic lower extremity arterial occlusions is effective without major hemorrhagic complications

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Eur J Vasc Endovasc Surg. 2014 Nov;48(5):551-8.
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Abstract

Objectives
To evaluate the efficacy and bleeding complications associated with a low-dose thrombolysis protocol for thromboembolic lower extremity arterial occlusions.

Design
A retrospective cohort study.

Materials and methods
A retrospective analysis was performed using data from all consecutive patients who underwent catheter-directed, intra-arterial thrombolysis for thromboembolic lower extremity arterial occlusions between January 2004 and May 2013. All patients were treated on a standard surgical ward. Endpoints were incidence of bleeding complications, duration of thrombolysis, angiographic patency rate, 30-day mortality rate and amputation-free rate at 6 months.

Results
Of the 171 cases analysed, 129 cases underwent low-dose thrombolysis and 42 cases underwent high-dose thrombolysis. No major bleeding complications occurred in the low-dose group vs. 5% in the high-dose group (P=.01). The median duration of thrombolysis was 67h (4-304h) in the low-dose and 49h (2-171h) in the high-dose group (P=.027). Angiographic patency was restored in 67% of the cases in the low-dose group vs. 79% of the high-dose group (P=.17). The 30-day mortality rates were 1% in the low-dose vs. 5% in the high-dose group (P=.09). However, this higher mortality rate was not related to bleeding complications. Major amputation-free rates at 6 months were 81% in the low-dose group and 88% in the high-dose group (P=.22).

Conclusions
Based on this data series, low-dose thrombolysis for thromboembolic lower extremity arterial occlusions is as effective as high-dose thrombolysis. By contrast, the risk of major bleeding complications is substantially lower when using low-dose thrombolysis.

Introduction

Prior to the 1990s, the standard treatment for acute leg ischemia was surgical thromboembolectomy. The publication of several prospective randomized trials in the 1990s showed that thrombolysis might represent an effective alternative to primary surgical intervention[1-2]. Since these landmark trials, a consensus has been reached that thrombolysis can be viewed as a first-line treatment for many cases of thromboembolic lower extremity arterial occlusion[3-5]. While a range of fibrinolytic agents and a variety of dose protocols have been used for thrombolysis6, most studies have reported results of urokinase (UK) at doses of more than 100 000 IU/h, together with varying doses of heparin. These studies reported major bleeding rates ranging from 6% to 13%, including 2% intracranial bleeding, and minor bleeding complications in 5% to 17% of patients[1,2,7,8]. Overall success rates for high-dose thrombolysis are reported to be around 70% (Table I).

Before 2011, a low-dose thrombolysis protocol consisting of a 500 000 IU UK intra-arterial bolus, followed by continuous infusion of 50 000 IU UK/h and 4 800 IU of heparin per 24 hours was routinely used in our university hospital. In mid-2011 this protocol was replaced by a high-dose protocol (100 000 IU UK/h and 9 600 heparin/24h). Two factors triggered this decision: a nationwide survey on thrombolysis practice revealed that most Dutch hospitals use high-dose protocols, and our successful use of a high-dose protocol during a clinical trial to evaluate ultrasound-accelerated thrombolysis compared to standard thrombolysis. However, the safety and effectiveness of high-dose thrombolysis was called into question in our hospital following two incidents of major bleeding complications in rapid succession. This was the rationale underlying our decision to retrospectively evaluate thrombolysis success rates and bleeding complications of both our low- and high-dose thrombolysis protocols.

Materials and methods

This retrospective analysis included data from all consecutive patients who underwent thrombolysis for thromboembolic occlusions of native arteries or bypass grafts distal to the aortic bifurcation in the period January 2004-May 2013. We have acquired approval of the institutional ethics review board. The results for patients treated with low and high-dose protocols were analysed separately.

Clinical and outpatient records, radiological reports, surgeons' and nurses' reports were all reviewed. Patients were excluded on the basis of a thromboembolic occlusion directly caused by an endovascular intervention and when treated with an EKOS EndoWave infusion catheter system9, since patency rates, lysis duration, doses of urokinase and heparin and therefore risk of haemorrhagic

Table I. High-dose urokinase thrombolysis protocols

Author	N	Urokinase dose	Heparin dose	Success rate	Major bleeding	Intracranial bleeding	Minor bleeding	Amputation-free rate	Mortality
Cragg 1991[8]	35	250 000 IU bolus 250 000 IU/h for 4 h 125 000 IU/h up to 24 h	Intravenous heparin: 2-3 x APTT	77%	6%	none	17%	92% at 30 days	2% at 30 days
STILE 1994[2]	66	250 000 IU bolus 240 000 IU/h for 4 h 120 000 IU/h up to 36 h	5 000 IU bolus + 1 000 IU/h intravenous heparin: 1.5-2 x APTT + intra-arterial heparin following institutional protocol	75%	6%	2%	5%	87% at 6 months	4% at 30 days 8% at 6 months
Ouriel 1998[1]	272	240 000 IU/h for 2 h 120000 IU/h up to 48 h	intravenous heparin: 1.5-2 x APTT (>only first 67 patients, hereafter: subtherapeutic)	68%	13%	2%	5%	72% at 6 months	16% at 6 months
Duda 2001[7]	70	25 000 IU bolus per 10cm thrombus 240 000 IU/h for 2 h 120 000 IU/h for 2 h 240 000 IU/h for another 2 h	50 IU/kg bolus + 7 IU/kg/h intravenous heparin	70%	6%	none	13%	88% at 6 months	8% at 6 months

N = Number of cases

complications are probably influenced by this new thrombolysis technique. We followed recommendations in the literature and defined occlusions in patients with symptoms of less than 14 days duration as acute and those with symptoms of 14 days or more as non-acute[2].

In our hospital, thrombolysis is only performed as a primary treatment for suspected thromboembolic peripheral arterial occlusions in patients with viable extremities, i.e. not in immediately threatened limbs (Rutherford IIb/III) or in patients without evident pre-existing arterial occlusive disease presenting with hyperacute ischemia suggesting an embolic cause. Contra-indications for thrombolysis were active internal bleeding, recent (<10 days) surgery or trauma, recent (<1 month) peptic ulcer or gastrointestinal bleeding, oesophagus varices, recent (<3 months) intracranial bleeding, intracranial tumor, aneurysm or malformation, recent (<1 month) cardiopulmonary resuscitation, thrombocytopenia (<150×109/L) and coagulation disorders. After ipsilateral antegrade puncture or contralateral retrograde puncture under ultrasound guidance, an intra-arterial thrombolysis catheter (Royal Flush High-Flow, Cook Medical®, Amsterdam, the Netherlands) was advanced via a guide wire and placed into the proximal end of the thrombus or as close as possible. The standard thrombolysis protocol consisted of a 500 000 IU UK (Medac GmbH, Hamburg, Germany) lacing dose, followed by continuous infusion of 50 000 IU/h UK. A continuous dose of 4 800 IU/24h of heparin was infused through the side-port of the sheath to prevent peri-catheter clotting. After thrombolysis, patients were routinely heparinized and oral anticoagulant treatment was started.

In the second half of 2011, the low-dose protocol was changed to a high-dose protocol for reasons mentioned earlier. This protocol consisted of a 500 000 IU UK lacing dose, followed by continuous infusion of 100 000 IU/h UK together with a continuous heparin dose of 9 600 IU/24h, i.e. a doubling of continuous doses of urokinase and heparin. Decisions regarding follow-up angiographies, continuation of therapy and additional procedures were made by a dedicated team of vascular surgeons and interventional radiologists. The number of follow-up angiographies per 24h depended on the severity of ischemia and progression of thrombolysis. Patients were routinely treated on a standard surgical ward. All nurses and clinical residents involved in treatment underwent extensive training and had both paper and electronic access to the thrombolysis protocol at all times.

Haemoglobin (Hb), thrombocytes, Activated Partial Thromboplastin Time (APTT, normal range 25-40s), Prothrombin Time measured as International Normalized Ratio (INR, normal range 0.80-1.20) and fibrinogen levels (normal range 200-400 mg/dL) were monitored daily. In cases with a fibrinogen level below 100 mg/dL, the UK dose was halved and the fibrinogen level was checked after 3h. If the fibrinogen

level dropped below 50 mg/dL, UK infusion was stopped and the catheter perfused with NaCl 0.9%. After a period of 3h the fibrinogen level was checked again and therapy was continued if the fibrinogen level had recovered to >100 mg/dL. During therapy, aspirin was continued but no coumarines, low molecular weight heparins or intravenous heparins were administered. The handing of patients with coumarines/warfarin use pre-lysis included pro-thrombin time (INR) monitoring at the moment of admission. When pro-thrombin time was supratherapeutic (INR>3.5) patients received vitamin K and therapy was initiated when INR<2.5. After thrombolysis patients were routinely heparinized and oral anticoagulant treatment with coumarines was started concomitantly with a target INR range 2.5-3.5.

Thrombolysis was considered successful when angiographic patency was restored, i.e. restoration of luminal continuity without significant residual thrombus. Intra and retroperitoneal bleeding, intracranial bleeding and all bleeding complications requiring blood transfusion or invasive procedures were considered potentially life threatening and therefore categorized as major bleeding complications. Minor bleeding complications were defined as bleeding at any other site not requiring blood transfusion or invasive treatment.

The data were analysed using SPSS (IBM Statistics v20, Chicago, IL, USA). A Mann-Whitney-U test or an unpaired Student's t-test was used to compare continuous variables with (non) parametric distributions. A Chi-square test was used to compare proportions between groups. P-values <.05 were considered statistically significant.

Results

During the inclusion period, thrombolysis was performed for 276 cases of lower extremity arterial occlusions in 199 patients. A total of 171 cases were included, 129 cases in 103 patients treated with low-dose thrombolysis and 42 cases in 29 patients treated with high-dose thrombolysis. Reasons for exclusion are described in Figure 1. Characteristics of included patients and occlusions are summarized in Tables II and III, respectively. Baseline and occlusion characteristics were not significantly different between groups.

Table II. Baseline characteristics of patients

	Low-dose group (n=129)	High-dose group (n=42)	P
Age -(in years, mean ±SD)	64 (±12)	64 (±10)	.94
Sex –(% male)	57	67	.29
Vascular history – (%)	77	86	.38
Cardiac history – (%)	35	31	.75
Current tobacco use– (%)	57	61	.70
Hypertension – (%)	77	83	.37
Hyperlipidaemia – (%)	81	86	.47
Diabetes – (%)	23	36	.11
Tissue necrosis at hospital admission– (%)	14	10	.12
Duration of symptoms -(in days, mean ±SD)	8 (±11.8)	8 (±11.9)	.29

Table III. Occlusion characteristics

		Low-dose group (n=129) n (%)	High-dose group (n=42) n (%)	P
Acute		102 (79)	31 (74)	.48
Non-acute		27 (21)	11 (26)	
Native artery		69 (53)	17 (40)	.14
Bypass graft		60 (47)	25 (60)	
Venous graft		14 (23)	5 (20)	.29
Prosthetic graft		45 (75)	19 (76)	
Combined graft		1 (2)	1 (4)	
Location occluded segment	Aorto-iliac	24 (19)	13 (31)	.18
	Femoral	73 (56)	23 (55)	
	Popliteal	24 (19)	3 (7)	
	Crural	8 (6)	3 (7)	

Acute is defined as occlusion with symptoms existing less than 14 days. Non-acute is defined as occlusion with symptoms existing 14 days or more. N = Number of cases

Treatment characteristics and results

Treatment characteristics of both groups are summarized in Table IV. The median duration of thrombolysis was 67h (range 4-304h) in the low-dose vs. 49h (2-171h) in the high-dose group, P=.027, and the median frequency of follow-up angiographies was 1.0 (0.3-2.3) vs. 1.5 (1.1-4.0) per 24h, P<.001. Angiographic patency was restored in 87 cases (67%) in the low-dose vs. 33 cases (79%) in the high-dose group (P=.17). For the low-dose group, success rates increased in the second half of the inclusion period: in the period 2004-2007 patency was restored in 57% of the cases vs. 77% in the period 2008-2011 (P=.02).

Table IV. Treatment characteristics

	Low-dose group (n=129)	High-dose group (n=42)	P
Treatment duration – (in h, median + range)	67 (4-304)	49 (2-171)	.03
Total UK dose - (in million IU, mean ± SD)	4.7 (±3.1)	6.1 (±3.8)	.02
Angiography frequency – per 24h, median + range)	1.0 (0.3-2.3)	1.5 (1.1-4.0)	<.001
APTT - (in s, mean ± SD)	59 (±30)	65s (±52)	.29
INR - (ratio, mean ± SD)	1.5 (±0.4)	1.7 (±0.6)	.04
Fibrinogen - (in mg/dL, mean ± SD)	264 (±114)	204 (±65)	.002
Technical success rate – (%)	99	100	.75
Patency rate – (%)	67	79	.17
Major bleeding complications – (%)	0	5	.01
Minor bleeding complications – (%)	5	7	.39
30-day mortality rate – (%)	1	5	.15
6 months amputation-free rate – (%)	81	88	.32

The median period of admission of all patients was 9 days (2-147 days). Patients successfully treated with thrombolysis had a significantly shorter in-hospital stay, independent of dose regimen, than patients in whom thrombolysis failed; 8 (2-82 days) vs. 13 days (2-147 days), respectively (P=.001).

Success rates of thrombolysis for all cases were higher (although still non-significant) for acute compared to non-acute occlusions; 73% vs. 61% respectively (P=.14). However, the median duration of symptoms in patients successfully treated with thrombolysis compared to non-successfully treated patients was significantly shorter at 3 days (0-67 days) vs. 7 days (0-60 days), respectively (P=.006). The success rates of thrombolysis for occluded native arteries and bypass occlusions did not differ (70% vs. 71%), and thrombolysis of prosthetic bypasses was significantly

more successful than thrombolysis of venous bypasses at 77% vs. 53%, respectively (P=.04). All of the above outcome parameters were independent of dose regimen. Univariate analysis showed that a variety of factors including history of vascular interventions, cardiac history, diabetes, smoking, hyperlipidaemia and hypertension did not significantly influence thrombolytic success.

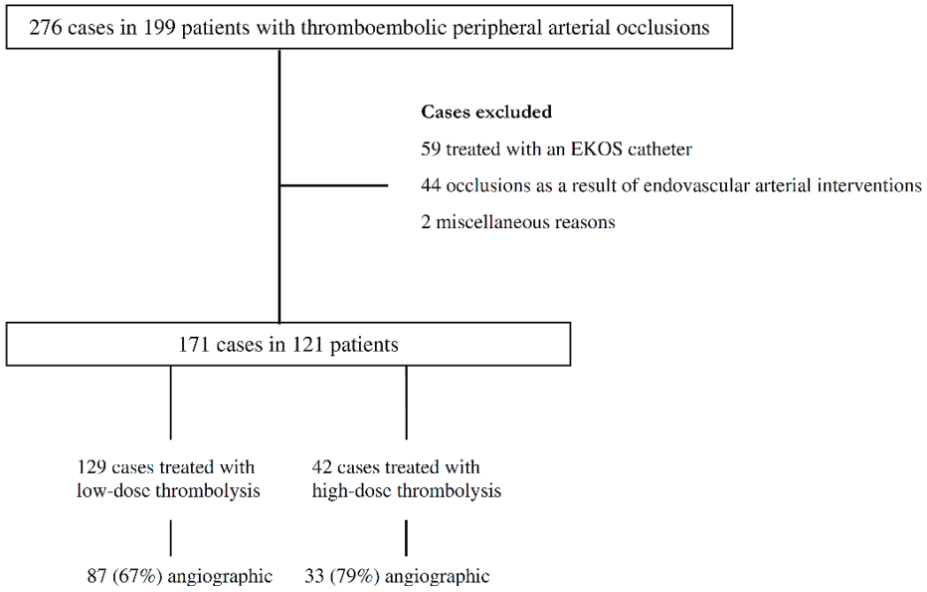


Figure 1. Excluded cases; values presented are cases.

Complications

Although no major bleedings occurred in the low-dose group, minor bleeding was noted in 6 cases (5%) and included bleeding at the puncture site in 5 cases and haematuria in a single case. Two of these six cases developed a groin haematoma, which led to the premature abortion of thrombolytic therapy. Other complications included delirium (2 cases), compartment syndrome of the leg (2), pseudoaneurysm formation (1), ischaemic stroke (1) and temporary kidney failure (1).

Two cases with major bleeding complications occurred in the high-dose group, including one case with intracranial bleeding and one with intra-abdominal bleeding. These cases resulted in a major bleeding complication rate of 5% in the high-dose group vs. 0% in the low-dose group (P=.01). The high-dose group also included 3 minor bleedings (7%), with bleeding at the puncture site, vs. 6 cases (5%) in the low-dose group (P=.39). Pseudoaneurysm formation of the femoral artery occurred in 1 patient in the high-dose group.

Follow-up of low-dose cases

Of the 87 cases successfully treated with low-dose thrombolysis, 42 (48%) underwent an additional percutaneous intervention and 8 (9%) underwent an additional surgical intervention (revision of bypass anastomosis, lumbar sympathectomy, toe amputation or below knee amputation) within the period of admission. The causes for unrestored patency in the remaining 42 cases were no or marginal lysis in 33, initial lysis followed by direct reocclusion in 4, abortion of therapy due to complications in 3 (catheter luxation due to a fall out of bed in 1 case, groin haematoma in 2 cases), technical failure (not possible to advance catheter) in 1 and non-compliance in 1 case. In 21 cases without restored patency, surgical revascularization was attempted (thromboembolectomy in 11 and bypass surgery in 10 cases; 5 of these cases underwent additional major amputation within 30 days) and direct major amputation was performed in 12 cases. One patient refused further treatment and in the remaining 8 cases, despite lack of restoration of patency in the target artery, thrombolysis resulted in clinical improvement and the patients could return home without any additional intervention.

Follow-up of high-dose cases

Of the 33 cases successfully treated with high-dose thrombolysis, 11 (33%) underwent an additional percutaneous intervention and 3 (9%) underwent an additional surgical intervention (revision of bypass anastomosis, toe amputation). In the high-dose group, 9 cases showed unrestored patency due to causes including no or only marginal lysis in 6, abortion of the procedure due to complications in 2 and initial lysis followed by direct reocclusion in 1 case. Major amputation followed for 4 of these 9 cases, and 2 cases underwent thromboembolectomy. Although patency of the target artery was not restored in 2 cases, thrombolysis resulted in clinical improvement and the patients could return home without any additional intervention. Finally, one patient underwent a second course of thrombolysis treatment.

Follow-up comparisons

Rates for 30-day mortality and remaining amputation-free for 6 months were not significantly different between low vs. high-dose groups, 1% vs. 5% and 81% vs. 88%, respectively (P=.09 and P=.22). In the low-dose group, the death of one patient was due to (pre-existing) heart failure, while one patient in the high-dose group died from (pre-existing) heart failure and a concomitant refusal of further therapy. An additional patient died two weeks after hospital discharge in good condition due to a traumatic subdural haematoma complicated by renal failure.

Laboratory values

Although the mean APTT values over the whole treatment period did not differ between groups (low vs. high) at 59 seconds (s) (±30s) vs. 65s (±52s) (P=.29), the

mean INR in the high-dose group was significantly higher at 1.5 (±0.4) vs. 1.7 (±0.6), (P=.04). Mean fibrinogen levels were also significantly higher in the low-dose group compared to the high-dose group, 260 (±110) vs. 200 (±60) mg/dL, respectively (P=.002). All cases with major and minor bleeding complications showed fibrinogen levels >100 mg/dL, and APTT and INR were within normal ranges on the day of the bleeding complication.

The APTT exceeded 60s at least once within the treatment period in 67% of the cases in the low-dose group vs. 55% in the high-dose group, indicating therapeutic treatment ranges despite the low-dose intra-arterial heparin administration. Furthermore, 51% of the cases in the low-dose group vs. 48% in the high-dose group showed an INR >2.0 at least once within the treatment period, indicating that INR levels were also within therapeutic ranges. Fibrinogen levels dropped below 100 mg/dL in 12% of the cases in the low-dose group (13 of 16 within the first 24h of therapy) and in 21% of the cases (4 of 9 within the first 24h of therapy) in the high-dose group. 2% of the cases in the low-dose group showed fibrinogen levels of ≤50 mg/dL vs. 7% in the high-dose group.

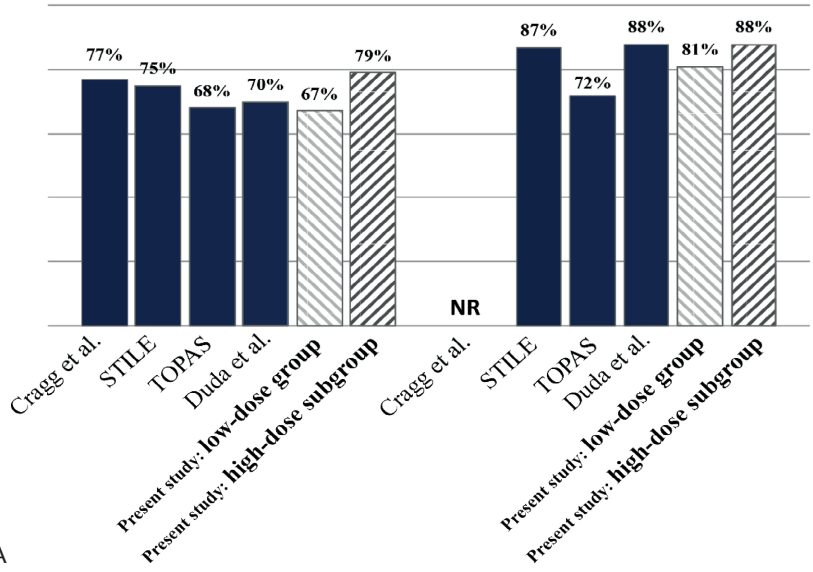
Discussion

An evidence-based guideline on optimal urokinase and heparin doses for the thrombolysis of thromboembolic peripheral arterial occlusions has not been produced to date. High-dose urokinase protocols are frequently used but are accompanied by high rates of major (6-13%) and minor (up to 17%) bleeding complications[1,2,7,8]. While the success rate of low-dose thrombolysis in the present study was comparable to the success rates of high-dose studies published in the literature (Figure 2A), the most significant finding in our study was that we achieved comparable angiographic patency rates without major bleeding complications (Figure 2B). Interestingly, our results using low-dose thrombolysis also improved over time, from 61% patency in the period 2004-2008 to 79% in the period 2009-2013 (P=.03), while baseline and occlusion characteristics remained stable. This improvement may be attributable to improved radiological skills. Overall, thrombolysis in prosthetic grafts was more successful than in venous grafts (77% vs. 53% respectively, P=.04), a finding also reported by several other studies[10,11]. This again raises the question of whether initial thrombolysis is the best therapy for occluded vein grafts.

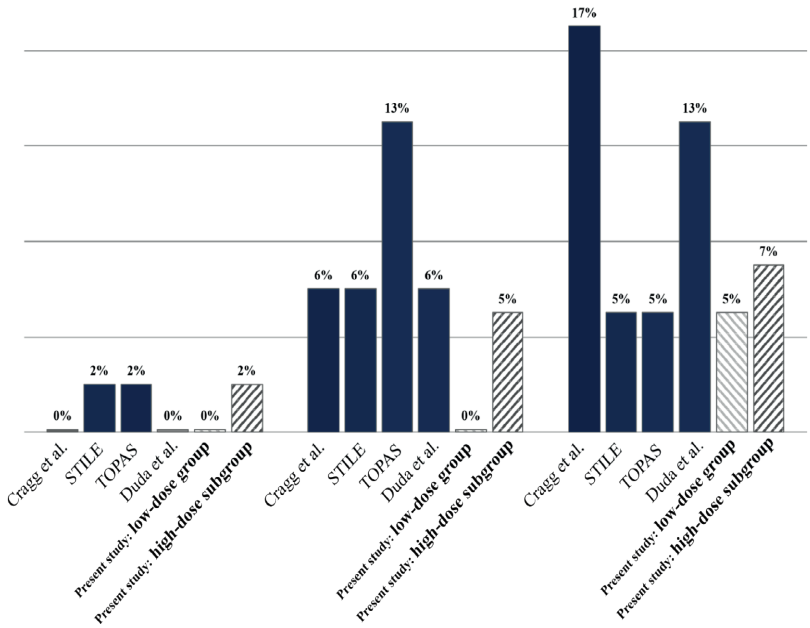
Similar rates of major bleeding complications were observed in our high-dose thrombolysis group when compared to literature, once again emphasizing the increased bleeding risk associated with treatment with higher doses of fibrinolytics and heparin. The overall 30-day mortality rate in this study was 2%, comparable to

the 2% mortality rate reported by Cragg et al. but lower than the 4% reported for the STILE trial[2,8]. The TOPAS trial did not report 30-day mortality rates.

Figure 2. Comparison of results with literature: (A) success rates, amputation-free rates at 6 months and (B) bleeding complications. NR = Not Reported



A



B

The low rate of bleeding complications in our low-dose group can be explained by the low-dose infusion of urokinase, as well as by the low-dose of the concomitantly administered heparin. Which of these factors contributes most to the favourable outcome cannot be determined from the present study. Despite the intended subtherapeutic intra-arterial administration of heparin to prevent peri-catheter clotting, APTT levels were within therapeutic ranges in the majority of patients at least once within the treatment period. This may be due to the potential synergistic effects of urokinase and heparin[12,13]. The concomitant use of unfractionated intravenous heparin during thrombolysis remains controversial[14,15]. In the TOPAS trial, the use of intravenous heparin (intended APTT 1.5-2 times the control value) was aborted after treatment of 62 patients, when the safety monitoring committee identified an unacceptably high rate (4.8%) of intracranial bleeding[1]. The initial requirement for therapeutic doses of systemic heparin was abandoned and replaced by subtherapeutic amounts of heparin, administered through the arterial sheath. This resulted in a drop in the rate of intracranial bleeding to 0.5%, suggesting a significant link between the co-administration of intravenous therapeutic doses of heparin and the risk of major bleeding. Another drawback of heparin use is the possible induction of heparin-induced thrombocytopenia thrombosis (HITT), which is rare but is associated with a high morbidity and mortality[16]. To our knowledge, a randomized trial of thrombolysis with and without concomitant heparin administration has not been performed. The necessity and safety of concomitant heparin infusion, whether intended as therapeutic or as subtherapeutic to prevent peri-catheter clotting and potential thrombus propagation, is questionable and should be further investigated.

The clinical use of laboratory tests during thrombolysis is controversial[17]. Although fibrinogen depletion was identified in the STILE trial as a risk factor for bleeding complications during thrombolysis with urokinase[2], a lack of other randomized controlled trials in the literature means that this is still an isolated finding. In our study, fibrinogen levels of patients with bleeding complications were all >100 mg/dL on the day of occurrence.

In the present study and in other studies, angiographic patency, i.e. restoration of luminal continuity, was used to define success of thrombolysis[1,2,7,8,18]. However, despite restoration of luminal continuity of the target artery, poor distal run-off might result in early reocclusion and thus hamper clinical improvement and indicate failed treatment. The opposite - clinical improvement without successful lysis of the target artery - might also occur and could be explained by lysis of a thrombus in important collateral or outflow arteries, leading to relief of ischemia. In 5% of our cases angiographic blood flow was restored but no clinical improvement was found, necessitating additional surgical therapy (bypass-revision, thromboembolectomy,

or major amputation). By contrast, in 4% of the cases clinical improvement was seen without restoration of luminal patency in the target artery.

In our study, thrombolysis was continued as long as progression of lysis was observed on follow-up angiographies, without clinical deterioration demanding a change of therapy. The median duration of therapy in the low-dose group was nearly 3 days. Other studies have described protocols that stop thrombolytic treatment at defined points such as 7, 24, 36 or 48h[1,2,7,8], although reasons for this strict discontinuation of thrombolysis at predefined time points were not reported. The relatively long duration of therapy in our study might be partly explained by the lower dose of urokinase. Furthermore, the frequency of follow-up angiographies might also influence therapy duration, with a higher frequency potentially resulting in earlier cessation of therapy. With the protocol change in 2011 with higher doses of urokinase we expected faster reperfusion. We anticipated on this with more frequent treatment evaluation with angiographies to prevent unnecessary overnight continuation of thrombolysis and thereby an unnecessary higher risk on haemorrhagic complications. Despite the longer therapy duration in the low-dose group, which might also influence limb salvage, the 6-month amputation-free rate of 81% comparable to that reported in the literature[1,2,7,8] (Figure 2A). ‘

Duration of ischemia is another factor that might influence success of thrombolysis and risk of major amputation. The STILE investigators found a non-significant trend towards an advantage for surgery compared to thrombolysis in the combined death and amputation outcome (9.9% vs. 17.8%; P=.08) in the group with symptoms lasting more than 14 days. However, in the group with symptoms of less than 14 days duration thrombolysis performed significantly better than surgery, 15.3% vs. 37.5% respectively (P=.01)2. This resulted in guidelines advising thrombolysis only for recent occlusive events, defined as symptoms lasting for a maximum of 14 days[17]. The patency rates of thrombolysis in the present study were (non-significantly) higher for acute compared to non-acute occlusions, 73% vs. 61% respectively (P=.14). In addition, patients successfully treated with thrombolysis compared to non-successfully treated patients had a shorter history of symptoms, 3 vs. 7 days, respectively (P=.006). In our data series, thrombolysis was successful in 5 out of 8 cases with occlusions with a duration of 1 month or longer. Patients with long-lasting occlusions who are unfit to undergo surgical intervention might therefore be considered candidates for thrombolysis. Additionally, as more than half of patients receiving thrombolysis in the STILE trial underwent reduced surgical procedures[2], thrombolysis might reduce the magnitude of additional interventions.

Potential bias could have been introduced by the comparison of non-contemporaneous groups. However, as time progressed patency results of low- and high-dose thrombolysis became more similar and we do not think the difference

in time could influence the higher risk on haemorrhagic complications with higher doses of urokinase. Although a limitation of this study remains the retrospective study design, we consider this study relevant due to the lack of available evidence for an optimal thrombolysis protocol and regimen for peripheral arterial occlusions and a high incidence of major haemorrhagic events.

Finally, due to the absence of major bleeding complications, performing low-dose thrombolysis on a general surgical ward appears to be safe. With an increasing number of patients undergoing thrombolysis, this option could reduce the logistic burden on special care units and might also lower the costs of therapy.

Conclusion

Based on our data series, low-dose thrombolysis for thromboembolic lower extremity arterial occlusions appears to be as effective as high-dose thrombolysis. In addition, low-dose thrombolysis results in a substantially lower risk of major bleeding complications.

Disclosures

No disclosures of competition of interests. There is no financial arrangement or relationship that could be construed as a conflict of interest.

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Chapter 3: Therapeutic use of microbubbles and ultrasound in acute peripheral arterial thrombosis: a systematic review

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Abstract

Catheter-directed thrombolysis (CDT) for acute peripheral arterial occlusion is time consuming and carries a risk of major hemorrhage. Contrast-enhanced sonothrombolysis (CEST) might enhance outcomes compared to standard CDT. In this study we systematically review all *in vivo* studies on contrast-enhanced sonothrombolysis in a setting of arterial thrombosis. A systematic search of the PubMed, Embase, Cochrane Library and Web of Science databases was conducted. Two reviewers independently performed the study selection, quality assessment and data extraction. Primary outcomes were recanalization rate and thrombus weight. Secondary outcome was any possible adverse event. The 35 studies included in this review were conducted in 4 different (pre)clinical settings: ischemic stroke, myocardial infarction, (peripheral) arterial thrombosis and arterio-venous graft occlusion. Due to high heterogeneity among the studies, it was not possible to conduct a meta-analysis. In almost all studies, recanalization rates were higher in the group that received a form of CEST. One study was terminated early due to a higher incidence of intra cranial hemorrhage. Studies on CEST suggest that adding microbubbles and ultrasound to standard intra-arterial CDT is safe and might improve outcomes in acute peripheral arterial thrombosis. Further research is needed before CEST can be implemented in daily practice.

Key words

Peripheral arterial thrombosis, microbubbles, thrombolysis, ultrasound, contrast-enhanced sonothrombolysis

Introduction

Peripheral arterial disease represents an affliction in which one or more non-coronary arteries are partially or completely occluded leading to compromised blood flow and eventually ischemia (Morcos et al. 2018). With rare exceptions, atherosclerosis remains the most common underlying pathology of this affliction. The estimated number of people that are affected ranges from 30-200 million worldwide (Morcos et al. 2018; Yurtkuran et al. 2013). Peripheral arterial disease in an advanced stage can cause acute limb ischemia (ALI), usually due to a thrombus that completely occludes a blood vessel. ALI is a life-threatening condition that may result in amputation or even death. Advancing age, chronic kidney disease and diabetes are all known risk factors for development of atherosclerosis. With an aging world population and a rising incidence of both chronic kidney disease and diabetes, peripheral arterial disease - and with it ALI - is likely to dramatically increase in the near future (Falluji and Mukherjee 2014). Rapid treatment is essential to restore blood flow to the extremity (Santistevan 2017).

In the majority of cases of ALI, treatment will consist of catheter-directed thrombolysis (CDT), with only a small percentage of patients (13,1% in a study by Davis et al, 2018) still needing primary surgical revascularization. With CDT, an intra-arterial catheter is guided to the site of the occlusion and used to administer the thrombolytic agent locally. Although CDT has the benefit of being minimally invasive, procedures are time consuming, expensive and carry a risk of major bleeding (Falluji and Mukherjee 2014). Current thrombolytic therapy is effective in many cases, but a simpler, safer and non-invasive treatment is urgently required to improve on patient outcomes and lower the burden for both the patient and the healthcare system. A new treatment currently being researched, is the addition of ultrasound-induced microbubble cavitation to standard CDT (sCDT), i.e. contrast-enhanced sonothrombolysis (CEST) (Unger et al. 2014). Microbubbles (MB), also known as ultrasound contrast agents, oscillate and collapse under the influence of ultrasound (US). The oscillations and cavitation cause highly energetic micro flows near the thrombus, thereby destructing the (surface) structure of the thrombus. This leads to a greater exposure of thrombus surface to the lytic agent and consequently can accelerate thrombolysis. Another development in CEST is the ability to load the microbubble with a lytic agent, such as tissue plasminogen activor (tPA) or urokinase. In addition, the arginine-glycine-aspartic acid-serine peptide (RGDS) can be added to the surface of the microbubble as a targeting ligand for thrombus (Nederhoed et al. 2017). Both developments might increase the efficacy of CEST even further, as the MB will adhere to the surface of the thrombus and the lytic agent can be released locally. Moreover, these factors might also reduce bleeding risks and cost of the treatment. CEST holds a considerable promise for therapeutic use in acute peripheral arterial thrombosis. Several studies have been

conducted on recanalization using microbubbles and ultrasound, however, their therapeutic use in peripheral arterial thrombosis has only been reported in a few studies (Ebben et al. 2015; Nederhoed et al. 2017). As very little research has been done on CEST in the setting of peripheral arterial thrombosis, we investigated the effectiveness and safety of CEST in any setting of arterial thrombosis, excluding articles on microvascular arterial thrombosis. The main objective of this systematic review is to give an overview of all relevant *in vivo* studies on the subject of CEST in arterial thrombosis and to raise awareness on the possibility of CEST amongst specialists treating acute peripheral arterial thrombosis. Our primary outcomes are recanalization rate or thrombus weight. Our secondary outcome is any adverse event that might be related to the given treatment.

Materials and methods

Search strategy and selection criteria

This study was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA)-statement (Liberatie et al. 2009). To identify all relevant publications on the therapeutic use of microbubbles in peripheral arterial thrombosis, three authors (one of whom an experienced research librarian) systematically searched the bibliographic databases PubMed, Embase, the Cochrane Library (via Wiley) and Web of Science for publications up to February 3, 2020. Search terms expressing 'microbubbles' were used in combination with search terms comprising 'thrombosis' and both controlled terms (MeSH in PubMed and Emtree in Embase) as well as free text terms (The Cochrane Library) were used. After the initial search, title and abstract of all citations were independently screened by two of the authors. Included were all studies that investigated therapeutic use of microbubbles and ultrasound in arterial thrombosis in an *in vivo* setting (human or animal) and that reported the outcome measures recanalization rate and / or thrombus weight. There were no limitations on language. Articles were excluded if they did not report original data, only consisted of case reports or where the full text was not available. As the focus of this review is CEST in peripheral arterial thrombosis, we also excluded articles where the therapeutic US was not directed at the occluded vessel, but at the outflow territory. All articles were screened for statements on approval of a local ethics committee, adherence to guidelines for animal care and/or obtaining informed consent. As articles that had no such statement might still contain valuable data this was not used as an exclusion criterion, but an annotation was added in the results section. For the complete search strategy see the appendix.

Quality assessment

Quality assessment of the included studies, was done independently by the two previous mentioned authors, using the SYstematic Review Centre for Laboratory

animal Experimentation (SYRCLE) tool for animal studies, the Methodological Index for Non-Randomized Studies (MINORS) criteria for non-randomized studies in humans and the Cochrane Risk of Bias tool for randomized trials in a clinical setting (Hooijmans et al. 2014; Slim et al. 2003; Sterne et al. 2019). Disagreements between the 2 reviewers were resolved via consensus meetings.

Data extraction

Two authors extracted the data following a pre-defined schedule that included: study design, subject studied (human or animal), number of subjects, location and duration of arterial thrombosis, type of microbubble used, dosage of microbubbles, site of injection (intravenous or intra-arterial), type of fibrinolytic agent used including dosage and site of injection, ultrasound settings, duration of therapy given, outcomes on recanalization rate and/or thrombus weight as well as on possible adverse events. When looking at the recanalization rate (primary outcome), the method for determining recanalization was noted as was the definition of successful recanalization. Mortality and intracranial hemorrhage (ICH) were scored separately in the possible adverse events (secondary outcome), as well as whether or not the ICH was symptomatic (sICH). Treatment protocols that combined the use of MB, US and a fibrinolytic agent were scored as the CEST arm of the study.

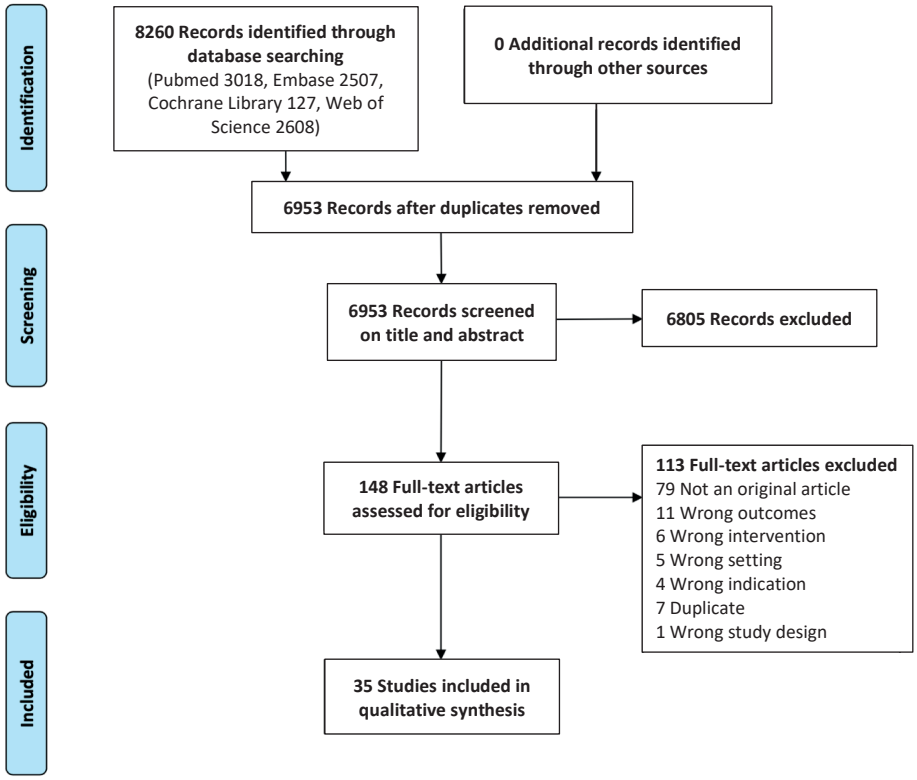

Figure 1. PRISMA flow diagram

Results

After removal of duplicates, 6953 records were identified for screening. Based on our inclusion and exclusion criteria, 35 studies were included in this review: 26 preclinical and 9 clinical trials, 3 of which were randomized. See **fig1** for the PRISMA flow diagram and **table 1** for an overview of the selected studies.

Quality assessment

Table 2 shows the results of the quality assessment. The preclinical studies all suffered from risk of bias due to problems in allocation generation and/or concealment. Baseline characteristics were unclear in most of these trials and -save for 1 study- caregivers and investigators were not blinded to the treatment given. Most studies did not report on the housing of the animals, but since these were short experiments this would have a minimal impact on the outcomes of those studies.

The preclinical models for arterial thrombosis that were used, were created to approximate four different clinical settings: ischemic stroke (n=8), myocardial infarction (n=4), arterial thrombosis (n=8) and arterio-venous graft occlusion (n=6). The clinical trials comprised 2 clinical settings: ischemic stroke (n=8) and acute myocardial infarction (n=2). There was a high heterogeneity in the selected studies. They not only varied in type of subject used for the experiments and in location of thrombus formation, but also in type of microbubbles used, the injection site of the microbubbles (intravenous or intra-arterial), duration of experiments, ultrasound settings, whether a fibrinolytic agent was used and if so, which fibrinolytic agent, the injection site of the fibrinolytic agent used, the method for monitoring outcomes and definition of success. It was therefore not possible to perform a meta-analysis on any of the predefined outcomes.

Primary outcomes

Recanalization rate

Definition of successful recanalization varied between the studies. Some defined success as (a variation of) any indication of flow in the previous occluded artery (10 studies), some used a complete return of flow or used varying degrees of TIMI (thrombolysis in myocardial infarction) /TIBI (thrombolysis in brain ischemia) or TICI (thrombolysis in cerebral infarction) scores (**table 3**) .

In the *preclinical trials*, twelve studies investigated a combination of microbubbles, ultrasound and a fibrinolytic agent (CEST). In 10 of the 12 studies, the rate of recanalization using CEST was higher than in any other tested arm (**table 4a1**). In the other 2 studies the recanalization rate was similar to the best results in another arm tested. One of these, the study by Tomkins et al. (2015), was the only study where no recanalization was found in the 10 occlusions studied with the combination of MB, US and a fibrinolytic agent. The investigators used platelet rich clots that seem to be highly resistant to thrombolysis in general. The other study that showed similar recanalization rates for CEST and the best compared alternative, was the study by Laing et al. (2012). They compared the thrombolytic effect of CEST to that of thrombus-targeted, tPA-loaded echogenic liposomes and found a comparable recanalization rate of 60% in both treatment arms.

The maximum recanalization rate in the groups combining MB, US and a fibrinolytic agent (CEST), excepting the study by Tomkins et al, varied between 56 and 100% (**table4a2**). In contrast, studies investigating the possibility of arterial recanalization using microbubbles alone (n=2) found 0% (0/6 and 0/10) recanalization rate. The effect of ultrasound alone, was investigated in 13 studies. Recanalization rates varied from 0% (5 studies) to 63% in the study by Gao et al. (2014 ; 5/8 occlusions, 4 complete). In this study, ultrasound was applied with a high MI of 2,4 and a short

pulse duration of 5μS. Ten studies tested the use of a fibrinolytic agent alone. The recanalization rate reported in these studies varied between 0% (2 studies) and 50% (1 study), with the majority of the studies reporting recanalization rates between 40 and 50% (6/10). The combination of US and a fibrinolytic agent, tested in 4 studies, showed recanalization rates between 20 and 50%. The combination of MB and a fibrinolytic agent, without addition of US, was only tested in the trial by Laing et al. (2012), previously mentioned. The protocol that was most frequently compared to CEST (17 studies), was the combination of US and MB. Looking at the 39 different protocols investigated in these 17 studies, recanalization rates varied from 0 (2 study arms)to 100% (5 study arms). The best recanalization rates of these studies varied between 29 and 100%.

Of the 12 preclinical trials that tested a form of CEST, only 2 administered the fibrinolytic agent intra-arterially as is common in sCDT for peripheral arterial thrombosis. The first of these was the study by Laing et al. (2012), mentioned previously. Their definition of success, was a return to at least 95% of baseline flow and they found a recanalization rate of 60% for both the group with echogenic liposomes and the high MI US CEST group. Ebben et al. (2015) studied sCDT vs CEST in a porcine model of peripheral arterial thrombosis. They found a median increase in flow of 27% in the CEST group and 0% in the sCDT group. If the same definition of success is used as in the study by Laing et al. (2012), their recanalization rate in the CEST group is 33% versus 0% using sCDT.

Clinical trials concerning CEST were performed in patients with ischemic stroke (8 studies) and myocardial infarction (1 study) (**table 4b**). There was a higher recanalization rate of the affected artery in the CEST group in all trials where CEST was compared to standard care in either another study arm or in previously reported data. All clinical trials investigated the use of an intravenous fibrinolytic agent as part of their thrombolytic therapy. Only Ribo et al. (2010) investigated CEST using intra-arterial tPA when recanalization did not occur with intravenous tPA alone (n=7 out of 16 patients) or when there was a contra-indication for intravenous thrombolysis (n=2). They found in-procedure recanalization in 7 of these 9 patients (2 complete, 5 partial).

Perren et al. (2008) compared the effect of MB combined with a 2-MHz transcranial color-coded duplex (TCCD) and intravenous tPA (n=11) vs the TCCD and tPA alone (n=15) in patients with acute MCA stroke. They found complete recanalization rate in 7 out of 11 patients in the CEST group (64%) at 1 hour treatment, vs 8 out of 15 in the control group (53%). They found a significantly greater improvement in clinical outcome in the CEST group vs the control (p = 0.05). In the study by Dinia et al. (2009), 188 stroke patients that received CEST were compared with a historic group of 98 stroke patients that received tPA + transcranial Doppler

(TCD). They found significantly higher recanalization rates in the CEST group at all measurement windows ($p < 0.05$) as well as a higher proportion of patients achieving independency at 3 months in the CEST group versus the control group (51 vs 37%; $p=0.052$). In the TUSCON (Transcranial Ultrasound in Clinical Sonothrombolysis) trial by Molina et al. (2009), the aim was to test the safety, tolerability and activity of various doses of perflutren lipid MB combined with TCD and i.v. tPA. It was a multicenter randomized trial that was terminated early due to safety issues (see secondary endpoint section). A total of 35 patients were randomized: 12 received 1,4ml of MB with TCD and tPA, 11 received 2,8ml of MB, and 12 received tPA with only brief TCD assessment (control group). Sustained complete recanalization at the end of the treatment, was 67% for the 1,4ml CEST group, 46% for the 2,8ml CEST group and 33% in the control group ($p = 0.255$). Functional independence at 3 months was 75% in the 1,4ml CEST group, 50% in the 2,8ml CEST group and 36% in the control group ($p = 0.167$).

One study compared 2 different MB (galactose-based air-filled vs sulphur hexafluoride based) combined with tPA and TCD in 138 MCA stroke patients (Rubiera et al. 2008). There were no differences in recanalization rate or clinical outcome. Slikkerveer et al. (2012) conducted a pilot safety and feasibility study of low dose tPA combined with US and MB prior to PCI in patients with acute myocardial infarction. All patients (n=10) received a single bolus of 50mg tPA in the ambulance. At presentation at the ER, they were randomized to either receive 1 vial of perflutren-containing lipid MB and high MI, short pulse duration US or a placebo. After 15 min, all patients underwent an emergency coronary angiogram and - when necessary - intervention. At angiography, 3 out of 5 patients in de CEST group had TIMI flow III versus 1 out of 5 in the control group ($p = 0.23$).

Thrombus weight

Only two studies reported on thrombus weight as outcome of the therapy, Ebben et al. in 2015 and Nederhoed et al in 2017. In the first study, standard intra-arterial CDT was compared to intra-arterial CDT enhanced with intravenous microbubbles and locally applied ultrasound. CEST resulted in a significant lower thrombus weight at the end of therapy in this study (1.1g vs 1.6g; $p = 0.01$). In the second study, the microbubbles were loaded with urokinase and targeted to thrombus by adding RGDS to the microbubble surface. The targeted, urokinase-loaded microbubbles were administered intravenously and high MI ultrasound (MI 1.1) was applied at the sight of the occlusion. This form of CEST was compared to intravenously administered urokinase, combined with the same regimen of ultrasound. Thrombus weight at the end of therapy was significantly lower in the CEST group (0.94g vs 1.54g; $p = 0.017$).

Secondary outcomes

Out of the 26 preclinical studies, 11 did not report safety outcomes. Of the 15 studies that did report safety outcomes, only 4 found possible adverse events: Ebben et al. (2015) reported 1 distal embolization in the group that received microbubbles. Wu et al. (2015) found histologic evidence of focal hemorrhage in the infarct area in 3 out of 18 pigs, but there was no difference in incidence between the groups. Tomkins et al. (2015) reported 2 incidences of subarachnoid hemorrhaging due to incorrect catheter placement and 1 catheter dislodgement. Finally, Nederhoed et al. (2017) reported 1 re-occlusion during therapy in the CEST group, possibly due to distal embolization.

In the clinical studies, the main adverse event reported was intracranial hemorrhage (**table 5**). Some studies only reported on symptomatic, i.e. clinically important ICH. The TUSCON trial was a prospective randomized multicenter phase II trial of MB dose escalation combined with systemic thrombolysis in patients with ischemic stroke (Molina et al. 2009). The aim was to include 72 patients (48 targets and 24 controls), but the study was terminated after 35 inclusions due to a higher incidence of sICH in the 2,8ml MB dose group (3/11 patients in this group, vs 0/12 in the other 2 groups; p =0.028). There was no incidence of sICH in the 12 patients receiving the first tier of the dose escalation study, i.e. 1,4ml of MB.

The only other adverse events were reported by Slikkerveer et al. (2012), who tested the feasibility of treatment with low dose thrombolytics in combination with ultrasound and microbubbles prior to primary percutaneous coronary intervention in patients with a first acute ST-elevation myocardial infarction. They reported 2 incidences of minor leakage at the injection site of the catheters, 1 in each treatment arm of the study, and 1 non-sustained ventricular tachycardia in each treatment arm.

Discussion

This systematic review highlights a number of positive outcomes in terms of efficacy and safety of CEST, but a lot still remains unknown. Studies suffer from low numbers, lack of randomization, differences in CEST protocols including variation in US settings, location of arterial thrombosis, duration of therapy and fibrinolytic agent used. Studied outcomes and mode to determine outcome, as well as definition of success varied widely across the studies. Therefore, comparisons among the studies are challenging, and conclusions on what protocol of CEST would be best for our patient with acute peripheral arterial occlusion are difficult to draw. Despite the heterogeneity of the included studies, of the preclinical trials only 1 study showed no recanalization in any of the treatment arms and in 1 study, US did not seem to have an influence when thrombus-targeted echogenic liposomes were

used. In all other preclinical studies where CEST was investigated, recanalization rates were higher than in any of the control arms. All clinical trials that compared CEST to standard care, whether in their study or in previously reported data, show a higher recanalization rate of the affected artery in the CEST group. Where clinical outcomes are reported, they are better than in the standard group. This was even true for the study arm of the TUCSON trial that received the 2,8ml dose of MB and had a higher incidence of ICH (Molina et al. 2009).

Although there were few adverse events reported that might be attributed to the addition of microbubbles and ultrasound to standard thrombolysis in the included studies, adverse events have been reported in other studies. Roos et al. (2016) reported an unexpected high incidence of coronary vasoconstriction in their study on the role of sonolysis for reducing microvascular injury in patients with myocardial infarction. In this study, patients with acute myocardial infarction received an intravenous infusion of the MB Definity at 1,3ml/min. Diagnostic ultrasound (MI=0,18) was used to guide therapeutic high-MI, long pulse duration ultrasound (MI=1,3, pulse duration 20μs) to the site of the myocardial perfusion defect. The treatment was given for a maximum of 15 minutes prior to PCI, during which diagnostic and therapeutic US regimens were alternated at the rate of 15s per imaging mode. The inclusion target for this safety and feasibility study was set at 20, but the trial was aborted after 3 of the first 6 included patients showed vasoconstriction unresponsive to nitroglycerine in the affected artery on angiography. None of the patients that showed vasoconstriction, suffered any lasting noticeable effects. A subsequent study by the authors using a porcine model for acute myocardial infarction, showed a decrease in coronary artery diameter distal to the injury site after application of ultrasound. The authors conclude that long-pulse-duration ultrasound might cause coronary vasoconstriction distal to the culprit vessel location. In a study by Mathias et al. (2019), 100 first-STEMI patients were randomized to either receive short pulse-duration (<5μs) high MI US directed at different myocardial segments combined with infusion of Definity or only very low MI diagnostic US combined with Definity. They found a significantly higher pre-PCI recanalization of the infarct vessel in the high MI US group (24/50 vs 10/50; $p<0.001$) and reported no adverse events. This supports the theory that the vasoconstriction found in the study by Roos et al. might have been due to the long pulse-duration US. Since affected vessels in peripheral arterial disease have a larger diameter and tissues distal to the affected vessels are more resilient to ischemic conditions, possible temporary vasoconstriction due to ultrasound is not expected to cause a clinical problem in the setting of peripheral arterial thrombosis.

Future perspectives

This systematic review was conducted to assess the safety and efficacy of CEST in peripheral arterial thrombosis. As there are so few studies on this subject,

we added all *in vivo* studies that tested a form of CEST in combination with an arterial thrombosis. This lead to the current overview, where most studies are conducted in a setting of small cerebral or coronary artery occlusions. Although we cannot extrapolate the data directly to patients with peripheral arterial disease, this overview gives us direction to where we might go with future studies and -hopefully- with improving treatment for our patients. For future clinical studies, we would suggest infusing the MB and applying local US during the first hour of treatment, as this seems to already achieve success in many of the trials in this review. A longer duration of US would burden both the patient and the care system. Furthermore we would suggest US with a high MI of around 1 and switching between 'on' and 'off' settings every few seconds, to allow the MB to re-enter the area of the thrombus. We have conducted the MUST-trial, a clinical safety and applicability trial for contrast-enhanced sonothrombolysis in acute peripheral arterial thrombosis, using these settings (Ebben et al. 2017). Inclusion has just finished and preliminary data show promising results without major complications (Evers et al. 2019). We need to wait for definitive data, however, to draw more solid conclusions. Another aspect to take into account when determining US settings for clinical use of CEST, is the vasoconstriction mentioned previously when a long pulse duration US was used. This might not be a problem with peripheral arterial occlusion, but use of a short pulse duration of <5μs can be explored. Depending on the definitive results of the MUST trial, a large multicenter randomized trial comparing standard intra-arterial CDT and CEST will be set up in the near future. End points will include time to reperfusion of the affected limb, major and minor amputations, duration of hospitalization and any adverse event with emphasis on intracranial hemorrhage. Long term follow up is needed to detect any possible long term side effects of the experimental treatment. For the more distant future, aims might not only include a better clinical outcome and shorter hospitalization times, but also an even less invasive treatment for the patient with the acutely ischemic limb. As discussed before, MB can be used as a vehicle for medication. They can be targeted to adhere to thrombus and with the most recent developments they can now also be directed magnetically (Chen et al. 2019). Combining these attributes, it seems a catheter-free intra-arterial form of thrombolysis might very well be possible in the future. More research is needed however, before these theories can be translated to a clinical setting. As mentioned previously we excluded articles where the therapeutic US was not directed at the occluded vessel, but at the outflow territory. The reason for not including studies that focused on outflow territory, is that the focus of this review is on revascularization of the ischemic limb using CEST and it does not seem feasible to treat the arterial occlusion by directing therapeutic ultrasound at such a large outflow territory that is also far away from the occlusion. Treating the microvasculature in peripheral arterial occlusion with a combination of microbubbles and US might still be beneficial though. Several studies reported on a better microcirculatory perfusion after using MB, which

could help in prevention of no-reflow phenomenon after revascularization of an acutely ischemic limb (Leeman et al. 2012; Xie et al. 2009b). We speculate that MB might also play a role in diabetic foot ulcers, as a loss of microcirculation is one of the main contributing factors for both the development of ulcers and the difficult healing process after macro vascular optimization of blood flow.

Conclusion

Studies on contrast-enhanced sonothrombolysis show promising results with regard to recanalization rate and clinical outcome in acute arterial thrombosis. It was not possible to perform a meta-analysis due to the high heterogeneity among the studies. Nonetheless, data suggest that adding microbubbles and ultrasound to standard intra-arterial catheter-directed thrombolysis might be a safe way to improve outcomes for patients with acute peripheral arterial thrombosis. More research on safety and applicability in a clinical setting is needed before it can be implemented in daily practice.

Conflict of interest statement

The authors declare that they have no affiliations that could inappropriately influence (bias) their work.

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Tables

Table 1. Overview of included trials

a. Preclinical (part 1)

Study	Objective	Conclusion
Nishioka 1997	To investigate the enhancing effect of MB on the in vivo recanalization rate of thrombotically occluded rabbit iliofemoral arteries by transcutaneous US exposure	DDFP emulsion significantly enhances the clot-disrupting effect of low frequency US
Birnbaum 1998*	To examine the efficacy of arterial clot disruption by a noninvasive, nonlytic approach with intravenous administration of perfluorocarbon-exposed sonicated dextrose albumin (PESDA) and transcutaneous delivery of US alone	In vivo arterial clot dissolution can be achieved with intravenous MB and transcutaneous US delivery alone
Culp 2001	To develop a method to declot full-size arteriovenous dialysis grafts using intravenous perfluorocarbon-exposed sonicated dextrose albumin (PESDA) MB and low frequency ultrasound (LFUS)	Direct injection of PESDA with transcutaneous LFUS succeeds in lysing moderate-size clots and recanalizing thrombosed fistulas
Porter 2001	To examine the effectiveness of different ultrasonic frequencies in recanalizing thrombosed vessels in a simple attenuation model	Transcranial and transthoracic US in the presence of intravenous MB can improve flow to ischemic regions
Culp 2003	To demonstrate utility of FDA-approved MB for arteriovenous graft declotting, establish efficacy levels, and assess US parameters inclucing intensity and wave-mode applications for this purpose	LFUS with direct injection of MB is effective in lysing moderate-sized clots and recanalizing thrombosed arteriovenous grafts. It best succeeds at the higher range of intensity settings tested in PW mode.

a. Preclinical (part 2)

					Reported outcomes		
Number of treatments (subjects)	Location thrombosis	Age clot (min)	Microbubble	Fibrinolytic agent	Recanal-ization rate	Thrombus weight	Safety
34 (17) Rabbit	IFA	<30	EchoGen (dodeca-fluoro-pentane)	None	X	..	X
25 (13) Rabbit	IFA	<30	PESDA	None	X	..	X
26 (3) Canine	FAVG	≥60	PESDA	None	X	..	X
15 (17) Porcine	CA (LCX)	<30	PESDA	None	X	..	X
104 (5) Canine	FAVG	≥60	Optison	None	X	..	X

a. Preclinical (part 1) *(continued)*

Study	Objective	Conclusion
Culp 2004	To demonstrate that transtemporal LFUS used with MB tagged with a low-dose glycoprotein 2b/3a receptor antagonist can recanalize acutely thrombosed intracranial vessels	Intravenous platelet-targeted MB combined with transcranial LFUS can rapidly open acute intracranial thrombotic occlusions.
Xie 2005	To determine the effectiveness of lipid-encapsulated MB and US in recanalizing arteriovenous graft thrombi and the effect that tissue attenuation has on the succes rate	US and MB are capable of recanalizing acute arteriovenous graft thromboses. Higher intensities may be needed in the presence of tissue attenuation.
Tsutsui 2006	To determine the added value of simultaneous imaging of intravenously infused MB that are being used to dissolve an intravascular thrombus with therapeutic US	The use of TUS with intravenous MB has a high success rate in recanalizing deeply located thrombosed arteriovenous grafts when performed with DUS guidance.
Xie 2009a	To determine whether DUS, in the presence of MB, would be capable of recanalizing deeply located intravascular thrombi without the need for fibrinolysis or systemic anticoagulation	DUS can recanalize thrombosed vessels without a fibrinolytic agent, high MI US has a higher recanalization rate than low MI US
Xie 2009b	To determine whether a low-MI MB sensitive imaging system could be used to guide high-MI impulses from the same diagnostic transducer during a platelet-targeted MB infusion and improve both epicardial and microvascular flow in a pig model of acute coronary thrombosis.	Intravenous platelet-targeted MB combined with brief high-MI DUS impulses guided by contrast pulse sequencing improve both epicardial recanalization rates and microvascular recovery.

a. Preclinical (part 2) *(continued)*

Number of treatments (subjects)	Location thrombosis	Age clot (min)	Microbubble	Fibrinolytic agent	Reported outcomes: Recanalization rate	Reported outcomes: Thrombus weight	Reported outcomes: Safety
23 (15) Porcine	APA	120-420	PESDA	None	X	..	..
55 (4) Canine	FAVG	240	Definity	None	X	..	..
24 (1) Canine	FAVG	240	MRX815	None	X	..	X
24 (2) Canine	FAVG	240	MRX-801	None	X	..	..
45 Porcine	CA (LAD)	≥20	MRX-835 + MRX-802	Prouro-kinase	X	..	X

a. Preclinical (part 1) *(continued)*

Study	Objective	Conclusion
Laing 2011	To test whether tPA-loaded echogenic liposomes (TELIP) with US will confer thrombolytic efficacy similar (or superior) to that of other clinically used or proposed tPA treatment modalities for ischemic strokes.	The thrombolytic efficacy of tPA-loaded ELIP is comparable to other clinically described effective treatment protocols, while offering the advantages of US monitoring and enhanced thrombolysis from a sitespecific delivery agent.
Ren 2011	To address whether P1 polypeptide-loaded MB can prolong the 3 h therapeutic window in cerebral thrombosis and achieve a higher recanalization rate with a lower risk of cerebral hemorrhage.	TMB/LFUS is an effective and safe therapy for thrombolysis in a 6 h cerebral thrombosis rabbit model
Lui 2012*	To evaluate the efficacy of MB in transcranial Doppler ultrasound (TCD)-assisted urokinase thrombolysis.	The addition of MB enhanced the effects of transcranial Doppler ultrasound-assisted urokinase thrombolysis.
Xie 2013	To determine the type of cavitation required for successfully dissolving intravascular and microvascular thrombi in acute myocardial infarction and whether longer pulse duration therapeutic impulses (sustaining the duration of cavitation) could restore both microvascular and epicardial flow with this technique.	Although short pulse duration guided therapeutic impulses from a diagnostic transducer transiently improve microvascular flow, long pulse duration therapeutic impulses produce sustained epicardial and microvascular re-flow in acute myocardial infarction

a. Preclinical (part 2) *(continued)*

Number of treatments (subjects)	Location thrombosis	Age clot (min)	Microbubble	Fibrinolytic agent	Reported outcomes: Recanalization rate	Reported outcomes: Thrombus weight	Reported outcomes: Safety
59 Rabbit	AA	10	Intrinsically echogenic lipid microbubbles and Definity	tPA	X	..	..
47 (52) Rabbit	CCA	360	DPPG/DSPE-PEG-BTC HM microbubble	tPA	X	..	X
32 Rabbit	MCA	60	SonoVue	Urokinase	X	..	X
36 Porcine	CA (LAD)	≥20	MRX-801	tPA	X	..	..

a. Preclinical (part 1) *(continued)*

Study	Objective	Conclusion
Hagisawa 2013	To examine the enhancing effect of thrombus-targeted bubble liposomes (BLs) developed for fresh thrombus imaging during ultrasonic thrombolysis.	TIMI grade 3 flow was present in a significantly higher number of rabbits with USD and targeted BLs than rabbits with USD and non-targeted BLs, or with rtPA monotherapy.
Gao 2014	To determine whether skull attenuation would limit the ability of US alone to induce the type and level of cavitation required to dissolve thrombi and improve cerebral blood flow (CBF) in acute ischemic stroke.	Guided high-MI impulses from an US imaging system produce sustained improvements in ipsilateral and contralateral CBF after acute cerebral emboli.
Hua 2014	To investigate the thrombolytic efficacy of a novel type of MB carrying tPA which can bind to a fresh thrombus via the arginine-glycine-aspartic acid-serine (RGDS) polypeptide in vivo under the exposure of US.	Ultrasound-induced targeted tPA-loaded MB release is a promising thrombolytic method with satisfactory thrombolytic efficacy, lowered tPA dose and potentially decreased hemorrhagic risk.
Ebben 2015	To investigate the effect of additional US and MB on standard low-dose intra-arterial thrombolysis in a porcine model of extensive peripheral arterial occlusion.	The addition of contrast-enhanced US accelerated the thrombolytic effect of low-dose intra-arterial thrombolysis in peripheral arterial occlusions.

a. Preclinical (part 2) *(continued)*

Number of treatments (subjects)	Location thrombosis	Age clot (min)	Microbubble	Fibrinolytic agent	Reported outcomes: Recanalization rate	Reported outcomes: Thrombus weight	Reported outcomes: Safety
54 Rabbit	IFA	<30	Perfluoro-carbon filled lipid MB	tPA	X	..	..
24 Porcine	ICA, APA and rete	255	Definity	None	X	..	X
70 (40) Rabbit	IFA	30	HM perfluoro-propane/ polyethylene-glycol	tPA	X	..	..
10 Porcine	IFA	100	SonoVue	Urokinase	X	X	X

a. Preclinical (part 1) *(continued)*

Study	Objective	Conclusion
Wu 2015	To determine whether guided high mechanical index (MI) impulses from a diagnostic US transducer during an intravenous MB infusion could augment low-dose fibrinolytic therapy in treating acute myocardial infarction (ST segment elevation myocardial infarction, STEMI).	Guided high MI-induced MB cavitation from a diagnostic transducer added to lowdose tPA can immediately improve regional function and reduce infarct size in acute STEMI
Tomkins 2015	To investigate the effect on recanalization rates of tPA therapy alone or in conjunction with US and a new MB formulation (BR38) in a model with platelet rich clots.	These platelet rich clots were highly resistant to tPA with or without MB-enhanced sonothrombolysis.
Ren 2015*	To investigate the efficacy of transcranial color Doppler ultrasound (TCCS) combined with MB and rtPA for thrombolysis in vivo.	TCCS + MB combined with rtPA is a relatively effective approach for ischemic arterial thrombosis with an additive or synergistic effect.
Zhu 2016	To study the thrombolytic effect of low-frequency US combined with targeted urokinase-containing MB contrast agents on treatment of thrombosis in rabbit femoral artery; and to determine the optimal combination of parameters for achieving thrombolysis in this mode	The optimal parameters for thrombolysis were determined to be 1) an US frequency of 2.2 MHz and 2) a 90,000 IU/kg dose of urokinase. US exposure time (30 min vs. 60 min) had no significant effect on the thrombolytic effects

a. Preclinical (part 2) *(continued)*

Number of treatments (subjects)	Location thrombosis	Age clot (min)	Microbubble	Fibrinolytic agent	Reported outcomes: Recanal-ization rate	Reported outcomes: Thrombus weight	Reported outcomes: Safety
32 Porcine	CA (LAD)	≥20	MRX-801	tPA	X	..	X
30 Rodent	MCA	>480	BR38	tPA	X	..	X
30 Rodent	CCA	180-240	SonoVue	tPA	X	..	..
72 Rabbit	IFA	≥20	HM biotinylated urokinase and RGDS Targestar SA	Urokinase	X	..	..

a. Preclinical (part 1) *(continued)*

Study	Objective	Conclusion
Nederhoed 2017	To compare intravenously administered targeted MB incorporating Urokinase and locally applied US, with intravenous Urokinase and US alone	Minimally invasive thrombolysis using i.v. targeted MB carrying urokinase combined with ultrasound is feasible and might accelerate thrombolysis compared with treatment with urokinase and ultrasound alone.
Porter 2017	To examine the effect of different forms of DUS induced cavitation in restoring branch flow following carotid artery thrombosis in a porcine model of common carotid stenosis and thromboembolism.	High MI 20usec pulse duration impulses during a commercial MB infusion can be used to recanalize acutely thrombosed carotid arteries and restore downstream flow without anticoagulants. However, this effect is only seen with SC inducing impulses and not at higher mechanical indices, when a paradoxical reversal of the thrombolytic effect is observed.
Cui 2017*	To determine whether combining TUS with MB could accelerate thrombolysis at the in vitro and in vivo studies without the use of fibrinolytic drugs	Recanalization rates and flow scores in TUS + MB group were significantly higher than the control and TUS group
Chen 2019	To investigate the feasibility and efficacy of magnetically targeted MB-mediated sonothrombolysis for the treatment of obstructive thrombi	The recanalization rate, average blood flow velocity, and hindlimb perfusion in the red and white thromboembolic models were all significantly higher in the US + M-MB and US + M-MB + r-tPA groups than in the control and US + C-MB groups.

a. Preclinical (part 2) *(continued)*

Number of treatments (subjects)	Location thrombosis	Age clot (min)	Microbubble	Fibrinolytic agent	Reported outcomes: Recanal-ization rate	Reported outcomes: Thrombus weight	Reported outcomes: Safety
9 Porcine	IFA	100	SonoVue (RGDS targeted microbubbles)	Urokinase	X	X	X
38 (21) Porcine	CCA	20	Definity	None	X	..	X
36 Rabbit	IFA	120	Perfluoro-carbon filled lipid MB	None	X	..	..
40 (20) Rodent	IFA	60	Magnetic (streptavidin beads to lipid shell) and normal lipid MB's	tPA	X	..	..

1b. Clinical (part 1)

Study	Objective	Conclusion
Molina 2006	To evaluate the effects of administration of MB on the beginning, speed, and degree of middle cerebral artery (MCA) recanalization during systemic thrombolysis and continuous 2-MHz pulsed-wave TCD monitoring	Administration of MB induces further acceleration of US-enhanced thrombolysis in acute stroke, leading to a more complete recanalization and to a trend toward better short- and long-term outcome
Perren 2007**	To study whether transcranial color-coded duplex ultrasound (TCCD), combined with a second-generation phospholipid encapsulated sulphur hexafluorid microbubble ECA, accelerates i.v. rtPA-thrombolysis in the acute phase of MCA stroke more than TCCD ultrasound monitoring alone	ECA enhanced TCCD monitored rtPA thrombolysis is superior to TCCD monitored rtPA thrombolysis in terms of residual flow improvement as measured by TIBI, especially during the first 30 min. It is also superior in the immediate (24h) clinical improvement as measured by NIHSS. The rate of hemorrhagic transformation was not different between the two groups
Pagola 2007	To determine the timing of recanalization in basilar artery occlusion treated with systemic thrombolysis, MB, and continuous TCD monitoring	Combined treatment with i.v. tPA, MB, and continuous US in acute BAO leads to early recanalization in a significant number of patients; this is associated with favourable outcomes

1b. Clinical (part 2)

					Reported outcomes		
Number of patients	Location of thrombosis	Time to treatment (min)	Microbubble	Fibrino-lytic agent	Recanal-ization rate	Thrombus weight	Safety
111	MCA	156	Levovist	tPA	X	..	X
26	MCA	<180	SonoVue	tPA	X	..	X
20	BA	180 (<720)	Levovist	tPA	X	..	X

1b. Clinical (part 1) *(continued)*

Study	Objective	Conclusion
Alexandrov 2008	To test the feasibility and safety of novel lipid coated MB containing perfultren, that are consistent in size (1 to 2 Um) and more stable in saline solution	Perflutren MB reached and permeated beyond intracranial occlusions with no increase in sICH after systemic thrombolysis, suggesting feasibility of further MB dose-escalation studies and development of drug delivery to tissues with compromised perfusion
Rubiera 2008	To compare the effect of galactose-based air-filled MB (levovist) and sulphur hexafluoride-filled MB (sonovue) on recanalization and clinical outcome in sonothrombolysis for acute ischemic stroke	MB administration during sonothrombolysis is associated with a high recanalization rate. Recanalization rates, clinical course and long-term outcome are comparable when administering galactose-basedair-filled MB or sulphur hexafluoride-filled MB.
Dinia 2009	To investigate the risk of hemorrhagic transformation (HT) after MB-enhanced sonothrombolysis in acute stroke	Microbubble administration was associated with early recanalization and a high rate of hemorrhagic transformation but does not seem to increase the risk of symptomatic intracranial hemorrhage
Molina 2009	To study the safety, tolerability, and activity of perflutren-lipid MB MRX-801 2MHz TCD insonation as an adjuvant to iv tPA, and conduct a phase I-II safety dose escalation study	Perflutren lipid MB can be safely combined with systemic tPA and US at a dose of 1.4ml. Safety concerns in the second dose tier may necessitate extended enrollment and further experiments to determine the mechanisms by which MB interact with tissues

1b. Clinical (part 2) *(continued)*

Number of patients	Location of thrombosis	Time to treatment (min)	Microbubble	Fibrino-lytic agent	Reported outcomes: Recanal-ization rate	Reported outcomes: Thrombus weight	Reported outcomes: Safety
15	MCA	<180	Perflutren-lipid MB	tPA	X	..	X
138	MCA	178 (mean)	Levovist & SonoVue	tPA	X	..	X
286	ICA (38), MCA (248)	158 (<180)	Galactose-based MB	tPA	X	..	X
35	MCA (33), PCA (2)	126-139 (mean)	MRX-801	tPA	X	..	X

1b. Clinical (part 1) *(continued)*

Study	Objective	Conclusion
Ribo 2010***	To evaluate safety and efficacy on middle cerebral artery (MCA) recanalization of local MB administration during intra-arterial thrombolysis and continuous TCD monitoring	The combination of ultrasound and i.a. MB and tPA may be a strategy to enhance the thrombolytic effect and increase recanalization rates
Slikkerveer 2012	To determine the safety and feasibility of treatment with low dose thrombolytics in combination with ultrasound and microbubbles prior to PPCI to increase the epicardial recanalisation rates in patients with a first acute STEMI	No significant difference between treatment and control group in safety (minor adverse events 2/5 vs. 2/5, p = NS) and outcome (TIMI III flow 3/5 vs. 1/5 respectively, p = 0.23) was recorded. These results demonstrate that the study protocol is feasible in the acute cardiac care setting and safe during treatment and follow-up

1b. Clinical (part 2) *(continued)*

Number of patients	Location of thrombosis	Time to treatment (min)	Microbubble	Fibrino-lytic agent	Reported outcomes: Recanal-ization rate	Reported outcomes: Thrombus weight	Reported outcomes: Safety
18	MCA	175 (median)	Levovist	tPA	X	..	X
10	CA (LAD 7, RCA 3)	134-177	Luminity	tPA	X	..	X

* No statement was included on adherence to guidelines for animal care or obtaining permission from a local ethics committee. ** No statement was given on approval of a local ethics committee or obtaining informed consent. *** Approval was given by a local ethics committee, but no statement on obtaining informed consent was included in the article.
Abbreviations: IFA = iliofemoral artery, CA = coronary artery, LCX = left circumflex, FAVG = femoral arterio-venous graft, APA = ascending pharyngeal artery, LAD = left anterior descending, AA = abdominal aorta, CCA = common carotid artery, MCA = middle cerebral artery, ICA = internal carotid artery, RCA = right coronary artery, BA = basilar artery, PCA = posterior cerebral artery

Table 2. Results of quality assessment.

a. SYRCLE's tool

Study	Allocation generation/ application	Baseline characteristics similar	Allocation concealment	Blinding caregivers/ investigators	Blinding outcome assessor	Incomplete outcome data addressed	Free of selective outcome reporting	Free of other problems that could result in bias
Nishioka 1997	🔴	🟡	🔴	🔴	🔴	🟢	🟢	🟢
Birnbaum 1998	🟡	🟡	🔴	🔴	🔴	🟢	🟢	🟢
Culp 2001	🟡	🟡	🔴	🔴	🟢	🟢	🟢	🔴
Porter 2001	🟡	🔴	🔴	🔴	🔴	🔴	🟡	🔴
Culp 2003	🔴	🟡	🟡	🔴	🔴	🟡	🟢	🔴
Culp 2004	🔴	🟢	🔴	🔴	🔴	🔴	🟢	🔴
Xie 2005	🔴	🟡	🔴	🔴	🟡	🔴	🟢	🔴
Tsutsui 2006	🔴	🟢	🔴	🔴	🟢	🟡	🟢	🔴
Xie 2009a	🔴	🟡	🔴	🔴	🔴	🔴	🟡	🔴
Xie 2009b	🟡	🟡	🔴	🔴	🟢	🟢	🟢	🟢
Laing 2011	🟡	🟡	🔴	🔴	🔴	🔴	🟢	🟡
Ren 2011	🟡	🟡	🔴	🔴	🟢	🟢	🟢	🔴
Lui 2012	🟡	🟡	🔴	🔴	🟢	🟡	🟡	🟢
Xie 2013	🟡	🟡	🔴	🔴	🔴	🟡	🟢	🔴
Hagisawa 2013	🔴	🟡	🔴	🔴	🟢	🟢	🟢	🟢
Gao 2014	🟡	🟡	🔴	🔴	🔴	🟢	🟢	🟢
Hua 2014	🔴	🟡	🔴	🔴	🟢	🟢	🟢	🟢
Ebben 2015	🟢	🟢	🔴	🔴	🔴	🟢	🟢	🟢
Wu 2015	🟢	🟡	🔴	🔴	🟡	🟡	🟢	🔴
Tomkins 2015	🔴	🟡	🔴	🔴	🔴	🟢	🟢	🟡
Ren 2015	🔴	🟡	🔴	🟢	🟢	🟢	🟢	🟢
Zhu 2016	🔴	🟡	🔴	🔴	🔴	🟡	🟢	🟢
Nederhoed 2017	🟡	🟢	🔴	🔴	🔴	🟢	🟢	🟢
Porter 2017	🟡	🟡	🔴	🔴	🟢	🔴	🟢	🔴
Cui 2017	🔴	🟡	🔴	🔴	🔴	🔴	🟡	🟢
Chen 2019	🔴	🟡	🔴	🔴	🔴	🟡	🟢	🟢

b. MINORS criteria

Study	A clearly stated aim	Inclusion of consecutive patients	Prospective collection of data	Endpoints appropriate to the aim of the study	Unbiased assessment of the study endpoint	Follow-up period appropriate to the aim of the study	Loss to follow-up less than 5%	Prospective calculation of the study size	Additional criteria for comparative studies: An adequate control group	Contemporary groups	Baseline equivalence of groups	Adequate statistical analyses
Molina 2006	🟢	🟢	🟢	🟢	🟡	🟢	🔴	🟡	🟢	🟡	🟢	🟡
Perren 2007	🟢	🔴	🟢	🟢	🟡	🔴	🔴	🟡	🟢	🟢	🟡	🔴
Pagola 2007	🟢	🟢	🟢	🟢	🟡	🟢	🔴	🟡	-	-	-	-
Rubiera 2008	🟢	🟢	🟢	🟢	🟡	🟢	🔴	🟡	🟢	🟡	🟢	🔴
Dinia 2009	🟢	🟢	🟢	🟢	🟢	🟢	🔴	🟡	🟢	🟡	🟢	🟢
Ribo 2010	🟢	🟢	🟢	🟢	🟡	🟢	🔴	🟡	-	-	-	-

c. Cochrane Risk of Bias tool

Study	Randomization process	Assignment to intervention	Missing outcome data	Measurement of the outcome	Selection of reported results
Alexandrov 2008	🟡	🟢	🟢	🟢	🟢
Molina 2009	🟢	🟢	🟢	🟢	🟢
Slikkerveer 2012	🟢	🟢	🟢	🟢	🟢
Mathias 2016	🟡	🟡	🟢	🟢	🟢

Green = Low risk of bias; Yellow = Some concerns; Red = High risk of bias.

Table 3. Overview of classifications for flow and recanalization

	Grade		Definition
TIMI*	0	No perfusion	No penetration of contrast past the clot in the obstructed artery.
(angiography)	1	Penetration without perfusion	Contrast passes the occlusion, but there is no perfusion of the target area.
	2	Partial perfusion	Contrast passes the obstruction and reaches the target area, but slower than adjacent normal vessels.
	3	Normal perfusion	Normal flow through obstructed artery in comparison to adjacent vessels.
TIBI**	0	Absent	Lack of regular pulsatile flow signals despite varying degrees of background noise.
(transcranial Doppler)	1	Minimal	Systolic spikes of variable velocity and duration, absent flow during all cardiac cycles, reverberating flow.
	2	Blunted	Flattened systolic flow acceleration of variable duration compared to control, positive end diastolic velocity and pulsatility index <1,2.
	3	Dampened	Normal systolic flow acceleration, positive end-diastolic velocity, decreased mean flow velocities (MFV) by >30% compared to control.
	4	Stenotic	MFV>80cm/sec and velocity difference of >30% compared to control OR if both affected and comparison sides have MFV<80cm/sec due to low end-diastolic velocities: MFV>30% compared to the control side and signs of turbulence.
	5	Normal	<30% mean velocity difference compared to control, similar waveform shapes compared to control.

Table 3. Overview of classifications for flow and recanalization *(continued)*

	Grade		Definition
TICI*** (angiography)	0	No perfusion	No antegrade flow beyond the point of occlusion.
	1	Penetration with minimal perfusion	The contrast material passes beyond the area of the obstruction but fails to opacify the entire cerebral bed distal to the obstruction for the duration of the angiographic run.
	2	Partial perfusion	The contrast material passes beyond the obstruction and opacifies the arterial bed distal to the obstruction. However, the rate of entry of contrast into the vessel distal to the obstruction and/or its rate of clearance from the distal bed are perceptibly slower than its entry into and/or clearance from comparable areas not perfused by the previously occluded vessel, eg, the opposite cerebral artery or the arterial bed proximal to the obstruction. 2a. Only partial filling (<2/3) of the entire vascular territory is visualized. 2b. Complete filling of all of the expected vascular territory is visualized, but the filling is slower than normal.
	3	Complete perfusion	Antegrade flow into the bed distal to the obstruction occurs as promptly as into the obstruction and clearance of contrast material from the involved bed is as rapid as from an uninvolved other bed of the same vessel or the opposite cerebral artery.

* Thrombolysis in Myocardial Infarction. Adapted from: the TIMI Study Group, 1985. ** Thrombolysis in Brain Ischemia (Demchuk et al. 2001). *** Thrombolysis in Cerebral Ischemia (Higashida et al. 2003).

Table 4. Recanalization rates for CEST vs best alternative arm in same study

a1. Preclinical (part 1)

Author	Design	Number of occlusions	Ultrasound setting	Clot age (min)	Duration of therapy (min)
Xie 2009b	prourokinase i.v. vs low-MI US guided high-MI pulses + prourokinase i.v. + tMB i.v. vs low-MI US guided high-MI pulses + prourokinase i.v. + ntMB i.v.	45	low MI (0.2) 1,5MHz pulsed; high MI (1.9) 1.5MHz pulsed	≥20	30 (+ 60 follow up)
Laing 2011	i.a. saline vs i.a. free tPA vs i.a. tPA mixed with Definity vs tPA-loaded echogenic liposomes; randomization within these groups for high vs low MI US or no US	59	6MHz pulses high MI (0.4) 2min vs 6MHz intermittently low MI (0.2) 30min	10	2 vs 30
Lui 2012	urokinase i.v. + US vs urokinase i.v. + MB i.v. +US	32	2MHz pulse 0,252W/cm2	60	20 (+ 100 follow up)
Xie 2013	rtPA i.v. vs rtPA i.v. + MB i.v. + high MI short pulse US vs rtPA i.v. + MB i.v. + guided low MI long pulse US	36	1.6MHz long pulses; high MI (2.0), pulse length 5μs vs low MI (1.0), pulse length 20μs	≥20	30 (+ 60min follow up)
Hua 2014	no treatment vs tPA i.v. vs TUS vs TUS + tPA i.v. vs TUS + ntMB i.v. + tPA i.v. vs TUS + tPA-loaded tMB i.v. vs DUS + tPA-loaded tMB i.v.	70	2 MHz, intensity of 1.8 W/cm2 TUS; 2 MHz, MI 1.4 DUS	30	30

a1. Preclinical (part 2)

		Recanalization rate		
Determination of recanalization	Definition of recanalization	Best result CEST	Best alternative arm	p
angiography	Evidence of contrast flow through the site of occlusion and normal runoff of flow distal to the occlusion.	60% (tMB)	20%	NS
pulsed spectral doppler	≥95% recanalization	60% (tPA mixed with Definity, high MI US)	60% (targeted echogenic liposomes)	NR
TCD	If blood flow velocity was improved >30% or waveform improvement was greater than 1 grade according to the TIBI grading system	56%	31%	0.154
angiography	Not defined	83% (1.0 MI long pulse duration)	45%	<0.05
B-flow imaging (non-Doppler US)	Reappearance of the signal post treatment	80% (ntMB, fibrinolytic & US)	50% (fibrinolytic & US)	<0.05

4a1. Preclinical (part 1) *(continued)*

Author	Design	Number of occlusions	Ultrasound setting	Clot age (min)	Duration of therapy (min)
Ebben 2015	urokinase i.a. vs urokinase i.a. + MB i.v. + US	10	1.6MHz, high MI (1.2) pulses (5sec off, 1 sec on)	100	60 UK +/- MB followed by 120 UK alone
Wu 2015	1/2 dose tPA i.v. vs full dose tPA i.v. vs 1/2 dose tPA i.v. + MB iv + high MI US vs MB iv + high MI US	32	intermittent high MI (2.0)	≥20	30 (+ 60min follow up)
Tomkins 2015	tPA i.v. vs tPA i.v. + US + MB i.v. vs saline i.v.	30	3MHz, continuous, MI 1.7	>480	60 (+ 70min follow up)
Ren 2015	no treatment vs rtPA i.v. vs TCCS + MB i.v. vs TCCS + MB i.v. + full dose rtPA i.v. vs TCCS + MB i.v. + 1/2dose rtPA i.v.	30	1MHz transcranial color Doppler, MI 0.9	180-240	20?
Zhu 2016	urokinase-loaded tMB i.v. in different settings: A 3 × 2 × 2 factorial table was applied to create different combinations of ultrasonic frequencies (Factor A: 1.6 MHz, 2.2 MHz, 2.8 MHz), doses of urokinase i.v. (Factor B: 90,000 IU/Kg, 180,000 IU/Kg) and ultrasound exposure time (Factor C: 30 min, 60 min).	72	1.6 MHz vs. 2.2 MHz vs. 2.8 MHz	≥20	30/60 (+ 120 min follow up)

4a1. Preclinical (part 2) *(continued)*

		Recanalization rate		
Determination of recanalization	Definition of recanalization	Best result CEST	Best alternative arm	p
ultrasonic perivascular flow probe	Changes in flow (ml/min) as percentage of baseline (=flow after creating stenosis; formula: change in flow/baseline flow x100%)	27%*	0%	NR
angiography	Not defined	83%	50% (in 2 arms: full dose tPA and MB+US)	NR
laser Doppler flow	Return to ≥ 100% of baseline regional cerebral blood flow	0%	0%	NS
ultrasound + color doppler flow imaging	Not defined, but based on result section any grade ≥1 in author created grading system	100% (full dose tPA)	50% (tPA alone)	0.046
pulsed Doppler flowmetry	Recanalisation >15% of baseline flow	93% (2,2MHz, 90.000U, 30min)		NA

4a1. Preclinical (part 1) *(continued)*

Author	Design	Number of occlusions	Ultrasound setting	Clot age (min)	Duration of therapy (min)
Nederhoed 2017	urokinase-loaded tMB i.v. + US vs urokinase i.v.+US	9	1.6MHz, 3μs pulse duration, 24kHz pulse repetition frequency, MI 1.1	100	60 (+ 120min follow up)
Chen 2019	saline i.v. vs. US + MB i.v. vs US + magneticMB i.v. vs US + magneticMB i.v. + rtPA i.v.	40	2 MHz, MI 1.9	60	30

4a2. Range of recanalization rates for different study arms

Study arm (number of studies)	Range of recanalization rate (%)
CEST (12)	56-100
Placebo & no ultrasound (7)	0-10
Microbubbles alone (2)	0
Ultrasound alone (13)	0-63
Fibrinolytic agent alone (10)	0-50
US & MB (17)	0-100
US & Fibrinolytic agent (4)	20-50
MB & Fibrinolytic agent (1)	50

4a1. Preclinical (part 2) *(continued)*

		Recanalization rate		
Determination of recanalization	Definition of recanalization	Best result CEST	Best alternative arm	p
ultrasonic flow probe	Any recanalization	80%	25%	NR
Doppler US (mechanical index [MI], 0.18; frequency, 14 MHz)	Not defined	100%	95% (magnetic MB; 40% for regular MB)	NS (<0.05 compared to regular MB)

* If success was defined as 'any recanalization', the result would be 67% for CEST, 25% for the alternative arm. If defined as 'recanalization over 15% of baseline flow', the result would be 50% for CEST, 0% for the alternative arm.

4b. Clinical (part 1)

Author	Indication	Design (number of patients per group)	Randomization factor	Ultrasound setting	Duration of therapy (min)
Molina 2006	Ischemic stroke	tPA i.v. +US + MB i.v.(38) vs tPA i.v. + US (37) vs tPA i.v. (36)	Not randomized	continuous 2MHz TCD	120
Perren 2007	Ischemic stroke	tPA i.v. + US + MB i.v. (9) vs tPA i.v. + US (15)	Not randomized	2MHz TCCD, pulsed-wave mode, 189 mW/cm2	60
Pagola 2007	Ischemic stroke	tPA i.v. + MB i.v. + US (20)	Not randomized	continuous 2MHz TCD	120
Alexandrov 2008	Ischemic stroke	tPA i.v. + US + MB i.v. (12) vs tPA i.v. + US (3)*	MB vs no MB, ratio 3:1	2 MHz, power outputs <720 mW	120

4b. Clinical (part 2)

Determination of recanalization	Definition of success	Recanalization rate		p
		Best result CEST	Best alternative arm	
TCD	Partial when blunted or dampened signals appeared in a previously absent or minimal flow and complete if the end-diastolic flow velocity improved to normal or elevated values	71% after 2hrs (54% complete)	68% after 2hrs (41% complete; tPA i.v. + US)	0.65
TCCD	Partial when the TIBI grade evolved from 0–1 to 2–3 and complete with a final TIBI grade of 4 or 5	63.6% after 1hr (all complete)	53.3% after 1hr (complete)	NR
TCD	Partial when dampened signals appeared in a previously demonstrated absent or minimal flow, complete if the end diastolic flow velocity improved to normal or elevated values	20% after 1 hour (10% complete), 50% after 24hrs (all complete)		NA
TCD	Partial if the affected MCA segment with the worst residual flow grade pretreatment showed an improvement by 1 TIBI grade or more to TIBI grade 2 or 3, complete if TIBI flow grades recovered to grade 4 or 5 within 2 hours	83% after 2hrs (50% complete)	66.7% after 2hrs (none complete)	NR

4b. Clinical (part 1) *(continued)*

Author	Indication	Design (number of patients per group)	Randomization factor	Ultrasound setting	Duration of therapy (min)
Rubiera 2008	Ischemic stroke	tPA i.v. + Levovist MB i.v. + US (91) vs tPA i.v. + Sonovue MB i.v. + US (47)	Not randomized	1.97 MHz pulsed-wave, 8 KHz pulse repetition frequency, 385 mW/cm2 spatial peak temporal intensity, MI 0.24	120
Dinia 2009	Ischemic stroke	tPA i.v. + MB i.v. (188) + US vs tPA i.v. + US (98; historical control group)	Not randomized	2MHz pulsed-wave diagnostic transducers, 750 mW	120
Molina 2009	Ischemic stroke	tPA i.v. (12) vs tPA i.v. + 1.4ml MB i.v. (12) vs tPA i.v. + 2.8ml MB (11) i.v. vs tPA i.v. + 5.6ml MB i.v. (0) vs tPA i.v. + 11.2ml MB i.v. (0)**	MB + US vs no MB + US, ratio 2:1	continuous 2 MHz TCD, maximum 606 ±19 mW output	90

4b. Clinical (part 2) *(continued)*

Determination of recanalization	Definition of success	Recanalization rate		p
		Best result CEST	Best alternative arm	
TCD	Partial when TIBI 2 or 3 appeared in a previously demonstrated TIBI 1 pattern, complete if TIBI 4 or 5 were achieved	32.2% LV/35.6% SV after 1hr, 50.0% LV/46.7% SV after 2hrs, 63.8% LV/54.5% SV after 6hrs. (% complete not specified)		NA
TCD	Partial when blunted or dampened signals appeared in a previously demonstrated absent or minimal flow, complete if the end-diastolic flow velocity improved to normal or elevated values	32.2% after 1hr, 50% after 2 hrs, 63.8% after 6hrs, 74.3% after 12 hrs (% complete not specified)	21% after 1hr, 36.7% after 2hrs, 44.5% after 6hrs, 56.2% after 12hrs (% complete not specified)	<0.05
TCD	Partial if abnormal signals were still seen at the distal portion (TIMI grade II flow equivalent), complete when a normal waveform or a low-resistance stenotic signal appeared at the selected depth of insonation (TIMI grade III flow)	84% 1.4ml group after 2 hrs (67% complete), 45% 2.8ml group after 2hrs (all complete)	58% after 2hrs (33% complete)	NR (0.255 for complete recanalization)

4b. Clinical (part 1) *(continued)*

Author	Indication	Design (number of patients per group)	Randomization factor	Ultrasound setting	Duration of therapy (min)
Ribo 2010	Ischemic stroke	tPA i.v. + US (16) vs tPA i.a. +MB i.a. + US (7 after first receiving tPA i.v. with no improvement of flow, 2 direct i.a.)	Not randomized	continuous 2MHz pulsed-wave diagnostic transducer, < 750 mW	60min tPA i.v., <60min i.a.
Slikkerveer 2012	Myo-cardial infarction	MB i.v. + US + tPA i.v. +/- PCI (5) vs saline i.v. + tPA i.v. +/- PCI (5)	MB + US vs no MB + US	Intermittent high MI impulses (5s on, 5s off), 28Hz pulse repetition period, 1.25ms pulse duration, 1.5 Mpa peak rare faction pressure, 26 mW/cm2 pulse average intensity at maximum MI.	15

4b. Clinical (part 2) *(continued)*

Determination of recanalization	Definition of success	Recanalization rate		p
		Best result CEST	Best alternative arm	
TCD and angiograpgy	Partial if TICI II, complete if TICI III	78% after i.a. treatment (22% complete), 78% after 12hrs (56% complete)	56% after 1hr (25% complete)	NR
angiography	TIMI grade III in culprit vessel at PCI***	60%	20%	0.23

* Study was terminated early for administrative reasons, thus not reaching a predetermined sample size of 40; ** Study was terminated early due to a high rate of ICH in the 2.4ml MB dose group; ***No documentation of TIMI grade previous to intervention

Table 5. Adverse events in clinical trials (part 1)

				CEST			Ultrasound and Microbubbles		
Study	Clinical condition	Treatment	Number of patients	sICH (%)	ICH (%)	Mortality (%)	sICH (%)	ICH (%)	Mortality (%)
Molina 2006	Ischemic stroke	CEST vs sono-thrombolysis vs thrombolysis alone	111	1 (2,6)	9 (23)	..	..	..	..
Perren 2007	Ischemic stroke	CEST vs sono-thrombolysis	26	1 (9,1)	..	..	..	..	..
Pagola 2007	Ischemic stroke	CEST	20	0	..	7 (35)	..	..	..
Alexandrov 2008	Ischemic stroke	CEST vs sono-thrombolysis	15	0	3(25)	4 (33)	..	..	..
Rubiera 2008	Ischemic stroke	CEST (Levovist vs Sonovue)	138	4 (2,9)	28 (29,2)	13(9,4)	..	..	..
Dinia 2009	Ischemic stroke	CEST vs sono-thrombolysis	286	5 (2,9)	48 (26)	23 (12)	..	..	..
Molina 2009	Ischemic stroke	CEST (various doses of MB) vs thrombolysis	35	3 (13)*	3 (13)**	3 (13)***	..	..	..
Ribo 2010	Ischemic stroke	CESTa vs thrombolysis	18	1(11)	..	1(11)	..	..	..
Slikkerveer 2012	Myocardial infarction	CEST vs thrombolysis	10	0	..	0	..	..	..

* All sICH occurred in the 2,8ml dose MB group. ** Two of these ICH occurred in the 1,4ml dose group (17%) and one in the 2,8ml dose group (9%). *** All 3 deaths were in the 2,8ml dose group (30%).

(part 2)

Sonothrombolysis			Thrombolysis			No thrombolytic therapy			
sICH (%)	ICH (%)	Mortality (%)	sICH (%)	ICH (%)	Mortality (%)	sICH (%)	ICH (%)	Mortality (%)	Follow up
1 (2,7)	7 (19)	..	2 (5,5)	1 (16)	..	..	..	..	3 months
1 (6,7)	..	..	..	..	..	..	..	..	15 days
..	..	..	..	..	..	..	..	..	3 months
0	1 (33)	1 (33)	..	..	..	..	..	..	3 months
..	..	..	..	..	..	..	..	..	3 months
3 (2,1)	17 (19)	15 (15)	..	..	..	..	..	..	24 hours
..	..	..	0	0	0	..	..	..	3 months
..	..	..	NR	NR	NR	..	..	..	3 months
..	..	..	0	..	0	..	..	..	4 months

Two deaths were attributed to sICH, 1 to progression of the ischemic stroke. [a] Thrombolysis was given intravenously in 16 out of 18 patients, 7 of whom then proceeded to intra-arterial CEST as there was no recanalization . The remaining 2 patients had a contra-indication for IV thrombolysis and received primary intra-arterial CEST. NR = not reported.

Part II

Pre-clinical in-vitro and animal studies

Chapter 4: Contrast-enhanced sonothrombolysis in a porcine model of acute peripheral arterial thrombosis and prevention of anaphylactic shock.

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Abstract

Acute peripheral arterial thrombosis can be threatening to life and limb. Dissolution of the thrombus by local catheter-directed intra-arterial infusion of fibrinolytic agents such as urokinase, is the standard therapy for thrombosis; however, this method is time-intensive, and amputation of the affected limb is still needed in 10–30% of cases. Furthermore, thrombolytic therapy carries the risk of bleeding complications. The use of small gas-filled bubbles, or ultrasound contrast agents (UCAs), in combination with ultrasound has been investigated as an improved thrombolytic therapy in acute coronary and cerebral arterial thrombosis. The authors describe a porcine model of acute peripheral arterial occlusion to test contrast-enhanced sonothrombolysis approaches that combine ultrasound, UCAs and fibrinolytic agents and recommend a strategy for preventing severe allergic reactions to UCAs in the pigs.

Introduction

Acute peripheral vascular disease is most often caused by a thrombus blocking an artery. This can lead to acute lower limb ischemia, which is associated with high mortality rates, and with amputation of the affected limb within 30 d after hospital admission in ~10–30% of patients[1]. Dissolution of the thrombus, for example, by catheter-directed intra-arterial infusion of fibrinolytic agents such as urokinase proximal to the thrombus site, can restore blood flow to the limb. However, this technique is invasive and time-consuming, requires repeated angiography and carries the risk of major hemorrhagic complications, resulting in high morbidity and mortality rates[2]. Improved therapies are therefore needed.

Sonothrombolysis is a promising technique for the treatment of acute peripheral arterial occlusion that combines thrombolysis with ultrasound[3,4]. The efficacy of sonothrombolysis in small vessels can be further enhanced with the addition of ultrasound contrast agents (UCAs)[5–7]. UCAs consist of small gaseous microbubbles (1–10 μm) with lipid shells that oscillate under the influence of low-intensity ultrasound and even collapse at higher intensities. These oscillations result in mechanical forces on the clot surface, making the thrombus more susceptible to thrombolytics[8]. These particles therefore have been broadly investigated to improve thrombolytic therapy[9].

A therapeutic approach that combines the use of ultrasound, UCAs and fibrinolytic agents has not been described previously in subjects with acute thrombotic occlusion of large peripheral arteries. To assess the efficacy of contrast-enhanced sonothrombolysis (CEST) for the treatment of acute peripheral arterial occlusion, an appropriate animal model is needed. Because porcine and human coagulation and fibrinolytic systems have similar components[10], we developed a porcine model of CEST by modifying a previously described model of intra-arterial thrombolysis[11]. Here, we describe a technique for inducing acute peripheral arterial occlusion in pigs for the investigation of different CEST protocols and for preventing anaphylactic shock after administration of UCAs.

Technique

All procedures were done at the Animal Laboratory of the VU Medical Center under the direct supervision and support of a licensed veterinary staff. All procedures were done in accordance with both the Dutch national guideline for humane animal treatment (Code Of Practice Welzijnsbewaking van proefdieren, 2004) and the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The VU Medical Center Animal Ethics Committee approved all experiments and procedures carried out on the animals.

Twenty-three adolescent female Yorkshire pigs, weighing between 58 kg and 90 kg, were housed at the research facility for acclimatization and quarantine for 1 week before initiation of the experiments.

Anesthesia

We sedated the pigs with an intramuscular injection of 28 mg per kg body weight ketamine (Alfasan, Woerden, The Netherlands), 0.5 mg per kg body weight midazolam (Actavis bv, Baarn, The Netherlands) and 1 mg atropine (Pharmachemie, Haarlem, The Netherlands). We then induced anesthesia with intravenous (i.v.) injection of 20 mg etomidate (B. Braun, Melsungen, Germany), after which the pigs were intubated. When necessary for cannulation of the airway, we repeated the administration of etomidate. During the procedure, we maintained anesthesia with 1.5–2.0% isoflurane (Pharmachemie, Haarlem, The Netherlands) administered endotracheally and with i.v. 50 µg/h fentanyl (Hameln Pharmaceuticals, Hameln, Germany), 50 mg/h midazolam and 20 mg/h pancuronium (Organon, Oss, The Netherlands). We also administered i.v. 5 ml per kg body weight per h 0.9% NaCl. Tidal volumes were set at 10ml per kg body weight, with a frequency of 15–18/min, and were adjusted depending on capnography to maintain the CO2 concentration between 35 mmHg and 40 mmHg. We assessed arterial blood gases regularly for pH, partial pressure of CO2 (pCO2) and partial pressure of oxygen (pO2) using the IRMA TruPoint blood analysis system (ITCmed, Edison, NJ). We also measured blood pressure, heart rate, oxygenation and body temperature (Solar 8000 patient monitor, Marquette, GE Medical Systems, Milwaukee, WI) as well as temperature and microcirculation of the affected limb (laser Doppler probe; Periflux 4001 Master, Perimed AB, Järfälla, Sweden) during the entire procedure.

Creation of a stable thrombus

Via a midline laparotomy, we identified the left common and external iliac arteries and ligated the internal iliac artery. We measured blood flow in the iliac artery using an ultrasonic flow probe (Transonic-Systems Inc., Ithaca, NY). We created a stenosis in the external iliac artery by reducing the diameter of the vessel with a vascular tourniquet, decreasing the flow in the iliac artery by 50 ± 10% (Fig. 1). To promote adhesion of a thrombus to the vessel wall, we damaged the endothelium by clamping and declamping the iliac artery with a straight Kocher clamp (Heljestrand, Eskilstuna, Sweden) over a length of 4 cm. We then occluded the artery with the same clamps proximally and distally and injected 100 units of freshly prepared bovine thrombin (Calbiochem, EMD Millipore, Darmstadt, Germany) intraluminally. We removed the proximal clamp after 1 h and the distal clamp after 90 min. When persistent flow in the iliac artery was present, we once again occluded the vessel and administered another 100 units of thrombin. Once a thrombus formed, we left it to stabilize for 30 min.

In the first pig, time to creation of thrombus (TCT) was 295 min, after which the iliac vessel still was not completely occluded. TCT diminished during the study period, however, to 95 min in the last of the four subjects. The main reason for this shortening of TCT may be that we adjusted our technique to increase the amount of endothelial damage given; we increased the force applied to the vessel by fully closing the clamp ratchet when clamping and we increased the amount of time spent causing the endothelial damage. Another adjustment in the procedure was to release the proximal clamp for a short time after injection of the bovine thrombin to allow for extra blood to mix with the thrombin; this prevented the thrombin from replacing some of the blood when it was injected into the vessel, which might originally have made the thrombin less effective.

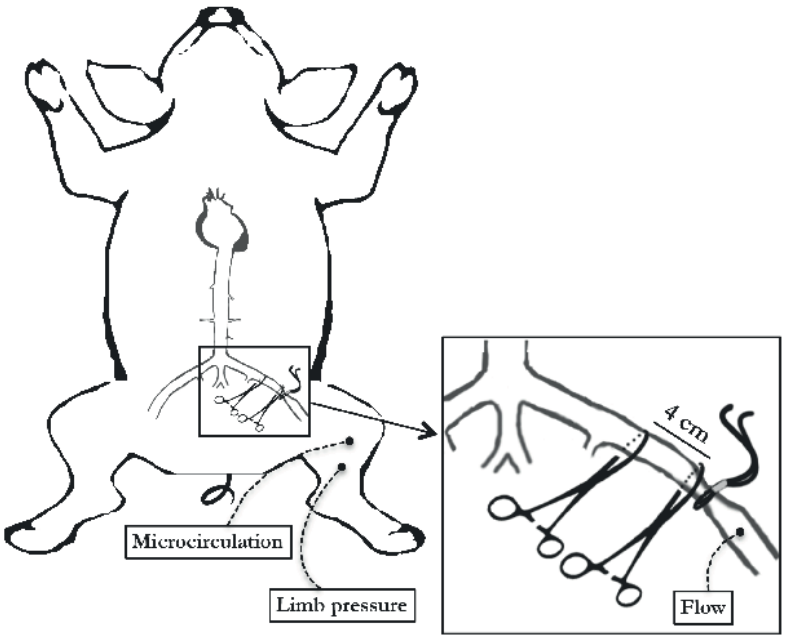

Figure 1. Diagram of the iliac artery. A stenosis was created, the endothelium was damaged and the artery was occluded. Illustrated by H.P. Ebben.

CEST procedure

To perform sonothrombolysis, we used a Philips Sonos 7500 ultrasound machine with a diagnostic S3 transducer (Philips Healthcare, Eindhoven, The Netherlands). We placed a balloon filled with saline between the probe and the arterial wall to mimic transcutaneous application of the probe, because, if direct contact with

the vessel was made, the probe would be too close to the thrombus to allow for visualization and treatment. The resulting distance between the ultrasound probe and the occluded vessel was 3 cm. We set the mechanical index (MI) to 1.1 with a focus of 3 cm and a frequency of 1.6 MHz.

From this point on, a variety of CEST protocols could be initiated. The UCA that we used was SonoVue (Bracco Imaging SpA, Milan, Italy), which consists of microbubbles with a phospholipidic monolayer shell containing sulfur hexafluoride. UCAs can be administered i.v. or intra-arterially , one vial per several minutes until the UCAs diminished from circulation, which can be controlled by ultrasound. The fibrinolytic agent that we used was urokinase (Lamepro bv, Breda, The Netherlands). Pigs received urokinase either i.v. or intra-arterially in a dosage regularly used in clinical setting: a bolus injection of 500,000 U followed by continuous infusion of 50,000 U per h. Microbubbles were administered i.v. and local ultrasound was applied to the occluded vessel. Because of the allergenic effects of the UCAs, therapy time varied from 0 to 180 min and all animals were terminated at the end of the experiment.

In the first pig that was administered UCA, systemic blood pressure dropped less than 2 min later, severe bradycardia occurred and ventilation was no longer possible owing to high airway resistance. We performed external cardiac compressions to no avail, and the pig died within 3 min. Differential diagnosis consisted of pulmonary embolism, anaphylaxis or severe hemorrhage elsewhere in the body. At autopsy there were no signs of pulmonary embolism. There was some intracranial hemorrhage, though not enough to be a likely cause of death. In the second, third and fourth pigs, similar reactions occurred a few seconds after each bolus of UCA, and external cardiac compressions were necessary in all pigs. Symptoms could not be prevented by administering i.v. methylprednisolone (Pfizer, Capelle a/d IJssel, The Netherlands) and tavegyl (Novartis, Breda, The Netherlands) 30 min prior to injection of the UCA, but the second, third and fourth pigs did respond to i.v. epinephrine (Pharmachemie, Haarlem, The Netherlands) in various doses (0.1–0.5 mg) and recovered. In these four pigs, severe hemodynamic changes and cardiac arrhythmia were caused by the anaphylactic shock, and the surviving pigs were excluded from further testing. We did not observe any cutaneous symptoms of allergic reaction in the pigs, and a literature search identified no previous reports of allergic reactions to UCAs in pigs. Allergic reactions to liposomes have been mentioned previously[12,13]; Szebeni et al.[14] recently stated that “nanoparticulate materials in pigs could lead to acute cardiopulmonary, hemodynamic, hematological, biochemical and dermatological changes within minutes, mimicking the human infusion (or anaphylactoid) reactions” and cited compliment activation-related pseudoallergy as a possible cause. Therefore, we found it necessary to premedicate the pigs prior to injection of the UCA to prevent possible allergic reactions to the

liposome outer layer of the microbubbles. Thirty minutes before injection of UCA in the remaining pigs, we administered 40 mg i.v. methylprednisolone and 500 mg indomethacin (Pharmachemie, Haarlem, The Netherlands) via rectal suppository. Because it takes time to obtain adequate blood levels of indomethacin when administered rectally, we also administered 2.5 g of i.v. acetylsalicylic acid (Sanovi-Aventis, Gouda, The Netherlands) in three doses, each given 5 min prior to administration of a dose of UCA in increments of 15 min. After introduction of the premedication step to the protocol, none of the remaining pigs (n = 19) experienced anaphylactic reactions.

Conclusions

Experiments to evaluate different protocols of CEST are currently being conducted using our porcine model, and results from these protocols are being analyzed. In view of our experience, we recommend that such experiments include premedication to prevent anaphylactic reactions to the lipid shell of the UCA. Our premedication approach was based on recommendations of other researchers in this field, who used an i.v. combination of 40 mg methylprednisone and 5 mg per kg body weight indomethacin. Since the cost of i.v. indomethacin (~$600 per 1-mg vial) is high, we instead used a suppository form of the drug (~$2 per 100 mg). Although this is not a new treatment in itself, indomethacin via suppository has not previously been described for this purpose to our knowledge. Using i.v. aspirin (~$190 per 500 mg) in a dose of 10 mg per kg body weight is another option with a lower cost than i.v. indomethacin and has previously been described as an effective method of preventing adverse reactions to liposomes[13]. Because aspirin has antiplatelet activity, there might be a slight effect on the outcome in a model of thrombolysis; however, patients with peripheral arterial vascular disease will almost always be taking antiplatelet medication. When comparing different methods of thrombolysis, the same systemic medications, including aspirin, should be used in both methods.

Competing financial interests

The authors declare no competing financial interests.

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Chapter 5: Therapeutic application of contrast-enhanced ultrasound and low-dose urokinase for thrombolysis in a porcine model of acute peripheral arterial occlusion.

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Abstract

Introduction
The addition of local ultrasound with contrast agents to standard intra-arterial thrombolysis has been promising in the setting of stroke and myocardial infarction. In this study, we investigated the effect of additional ultrasound and microbubbles on standard low-dose intra-arterial thrombolysis in a porcine model of extensive peripheral arterial occlusion.

Methods
In ten pigs, extensive arterial thrombosis was induced in the external iliac artery. The 'urokinase only' group (UK) (n=4) received standard thrombolytic therapy: intra-arterial bolus injection of 500,000 International Units (IU) followed by continuous low-dose urokinase (50,000 IU/h) infusion via an intra-arterial catheter and local intermittent application of ultrasound to visualize vascular patency. The 'urokinase + bubbles' group (UK+) (n=6) received the same urokinase therapy with concomitant infusion of microbubbles (5x 5mL vials during the first hour) intravenously and local intermittent application of ultrasound. After three hours of therapy the animals were terminated. End points were thrombus weight, arterial flow and microcirculation, representing reperfusion.

Results
Mean thrombus weights were 1.1 vs. 1.6 grams in the UK+ group and in the UK group, respectively (P=.01). Arterial blood flow increased in four out of six pigs in the UK+ group by a mean 61% versus one out of four in the UK group with 1%. Microcirculation and lower limb arterial pressure levels improved after start of therapy in the UK+ group, contrary to a trend of decline in the UK group. No signs of bleeding complications were observed in either group.

Conclusions
In this experimental pilot study, the addition of contrast-enhanced ultrasound improved low-dose intra-arterial thrombolytic therapy in extensive peripheral arterial occlusions. Further clinical studies are warranted.

Introduction

Acute peripheral arterial occlusions are limb- and potentially life-threatening. In western countries the incidence of patients with acute limb ischemia is around 140 per million per year, and rises every year[1]. In spite of rapid development of new endovascular techniques, in approximately 10-30% of the patients presenting with acute lower limb ischemia amputation follows within 30 days after hospital admission[2]. Since its introduction, intra-arterial thrombolysis has altered the treatment of acute peripheral arterial thrombosis. Randomized trials published in the 1990s showed that thrombolysis with urokinase might be a good alternative for primary surgical intervention[3,4]. Nevertheless, thrombolysis using fibrinolytic agents is time consuming and patients are confined to bed for days, which increases patients' burden. In the Netherlands we use the fibrinolytic urokinase, however, no standard protocol exists and different fibrinolytic agents with various dose protocols have been used throughout the years worldwide. Furthermore, thrombolytic therapy is accompanied by major bleeding complications in up to 9% of the cases with up to 3% intracranial bleeding[5], depending on dosage and length of treatment. However, low-dosed thrombolysis could be used to minimize bleeding complications. A disadvantage is that this regimen increases therapy time. Therefore improvement of thrombolytic therapy with low-dose fibrinolytics is needed to minimize bleeding complications and concomitantly reduce therapy time.

Thrombolysis can be enhanced by ultrasound (US), especially in the early phase of therapy[6]. In turn, the thrombolytic effect of fibrinolytics combined with US can be amplified with ultrasound contrast agents[7]. These encapsulated gas filled microbubbles (1-5μm) can pass freely through capillary systems without extravasation into the interstitial fluid. During exposure to high-intensity US, these bubbles tend to cavitate and collapse leading to formation of free radicals and microjets, causing mechanical stress, erosion of the clot and the formation of small holes in the clot surface[8]. These mechanical effects cause destabilization of the clot structure, making it more susceptible to fibrinolytics. Nowadays second-generation contrast-agents such as SonoVue (Bracco, Switzerland) and Definity (Lantheus Medical Imaging, MA, USA) are EMEA/FDA approved and safely used in clinical practice for diagnostic purposes. For therapeutic purposes such as thrombolysis, it has been applied in patients in clinical trials for the treatment of myocardial infarction and stroke[9,10].

The therapeutic application of microbubbles in thrombolysis has been investigated in animal models of myocardial infarction and stroke[11,12]. However, it has never been investigated in large peripheral arterial occlusions. We aim to improve the thrombolytic therapy of patients with peripheral arterial occlusions by reducing bleeding complications using low-dose thrombolysis with urokinase and

concomitantly shorten the therapy time with the use of microbubbles. Therefore in the present study we investigated the in-vivo application of this thrombolysis protocol in a porcine model of large peripheral arterial occlusion. End points were thrombus weight, arterial flow and microcirculation. We hypothesized that contrast-enhanced ultrasound can enhance low-dose thrombolysis in-vivo.

Methods

General protocol and anesthesia

Approval of the Animal Ethics Committee was obtained before initiation of the study.

Ten female adolescent Yorkshire pigs were housed at the research facility for a minimum of at least one week before initiation of the experiment to allow for quarantine and acclimatization. Animals were randomized (random choice of an animal by the animal caretaker) to either the control group (intra-arterial UK, designated as the UK group, n=4) or the intervention group (intra-arterial UK with intravenous microbubbles and local application of ultrasound, designated as UK+ group, n=6). The pigs are numbered in chronological order.

At the start of procedure, the animals were sedated with an intramuscular injection of ketamine 28mg/kg, midazolam 0,5mg/kg and atropine 1mg. Anesthesia was induced with 20mg of etomidate intravenously, after which intubation followed. Where necessary, the administration of etomidate was repeated to allow for cannulation of the airway. During the procedure, anesthesia was maintained with isoflurane 1,5-2,0% endotracheally, and fentanyl 50µg/h, midazolam 50mg/h and pancuronium 20mg/h intravenously. Furthermore 5 ml/kg/h of NaCl 0.9% were administered intravenously. Tidal volumes were set at 10ml/kg with a frequency of 15-18/min and adjusted depending on capnography, maintaining the CO2 concentration between 35-40mmHg. These initial parameters were modified after serial blood gas measurements to keep the pCO2 between 25 and 35 mmHg and the pH within normal limits.

All pigs (control group and intervention group) received 40mg methylprednisolone and 500mg indomethacin premedication and 2500mg acetylsalicylic acid in three doses during the procedure to prevent allergic reaction to the microbubbles[13].

An overview of the experimental protocol can be found in Figure 1. A catheter was placed in both the right carotid and femoral artery to measure blood pressure. Thermometers were placed in the esophagus and between the toes of both hind legs to measure core- and limb temperature. The electrocardiogram, blood

pressure, blood gases, urine output, transcutaneous oxygen saturation, and body temperature were monitored throughout the entire procedure.

Experimental surgical protocol

Via a midline laparotomy, the left common and external iliac arteries were identified and the left internal iliac artery was ligated. A stenosis was created in the left external iliac artery by reducing the diameter of the artery with a ligature, decreasing the flow in the iliac artery by 50%±10%. Proximal from the stenosis the endothelium of the arterial wall was mechanically damaged by clamping and declamping over a length of 4 centimeters of the external iliac artery in order to damage the endothelium and promote thrombus formation. Subsequently this artery was then clamped proximally and distally over a total length of 4 cm and 100 units (U) of bovine thrombin (Calbiochem, EMD/Merck, Germany) was injected intraluminally in order to create a thrombus. The proximal clamp was removed after one hour and the distal clamp was removed 30 minutes thereafter (Figure 1B). In case of persistent flow in the external iliac artery, the thrombus induction procedure was repeated by reclamping and additional injection of bovine thrombin. If necessary, small side branches were coagulated and large side branches were ligated. Blood flow in the external iliac artery distal to the occlusion was measured using an ultrasonic perivascular flow probe (T106, Transonic Systems Inc). A Laser Doppler probe (PeriFlux 4001 Master, Perimed Instruments) was placed transcutaneous on the affected limb in order to measure subcutaneous microcirculation.

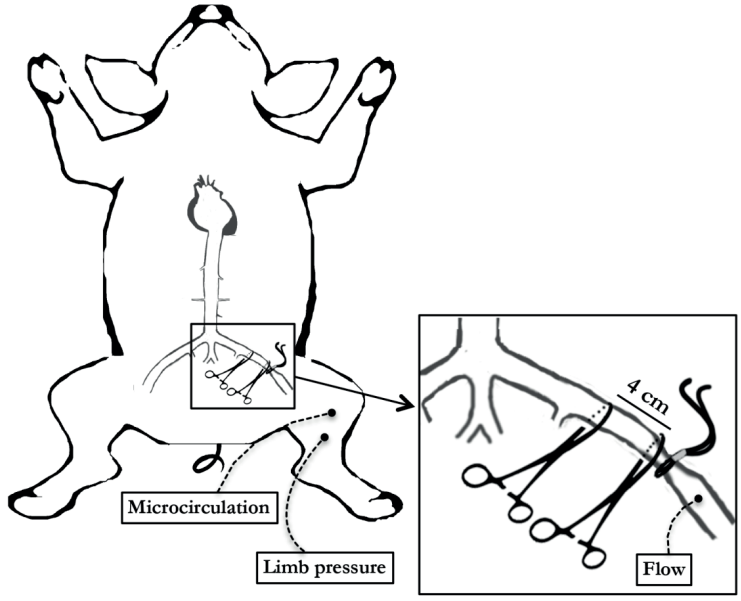
After thrombus induction (see above) and 10 minutes of thrombus stabilization, a thrombolysis catheter (Royal Flush High-Flow, Cook Medical®, Amsterdam, the Netherlands) was placed intra-arterially just proximal to the occlusion and thrombolysis was initiated. The catheter was placed using an antegrade approach via the common iliac artery in analogy to the standard intra-arterial thrombolytic treatment of peripheral arterial occlusions in patients. The control group, i.e. ' the UK group' received an intra-arterial bolus injection of 500,000 International Units (IU) of UK via the catheter, followed by the continuous low-dose infusion of 50,000 IU UK per hour. A diagnostic ultrasound probe (Philips Sonos 7500, 1.6 Mhz., focus: 3cm, MI: 1.2, S3 transducer, Philips, Best, the Netherlands) was directed at the external iliac artery at the site of the thrombus to visualize vascular patency during the procedure. To mimic transcutaneous application, the probe was placed on a balloon filled with saline resulting in a distance between the probe and the treatment artery of 3cm. In the 'UK+ group', the same UK infusion regimen was administered as in the UK group and the ultrasound probe was placed following the same protocol. However, in addition 5 vials (25mL) of microbubbles (SonoVue 5mL, Bracco, Switzerland, prepared following the manufacturer's manual) were infused via the ear vein during the first hour of thrombolysis. One vial was infused gradually

during 10 minutes, after an additional 5 minutes a new vial was infused. To ensure replenishment of the microbubbles in the treatment area, ultrasound impulses were applied intermittently (5 seconds off, 1 second on) until all microbubbles were destroyed at the site of the occlusion.

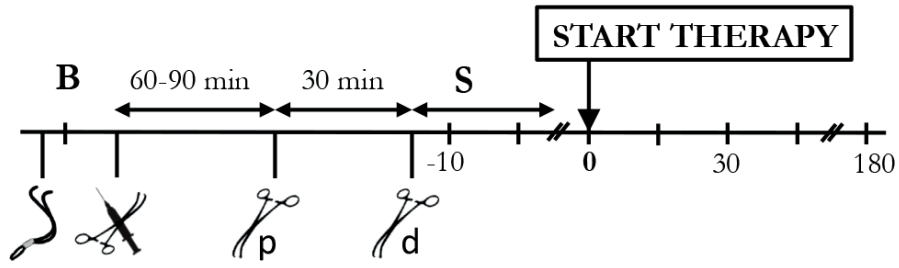
After 3 hours of thrombolytic therapy the pigs were terminated and autopsy was performed. Brains, kidneys, liver, lungs, heart and spleen were cut in thin slices and macroscopically inspected for potential (hemorrhagic) adverse events and tissue samples were taken. The left external iliac artery was excised and the persisting thrombus and 4 cm surrounding external iliac artery to which it adhered were weighed together. As control, four centimeters of the untreated right external iliac artery were excised and weighed.

The data were analyzed with SPSS (IBM Statistics v20, Chicago, IL, USA). A Mann-Whitney-U test or an unpaired Student's t-test was used to compare continuous variables with (non-) parametric distributions. A Chi-square test was used to compare proportions between groups. A P-value of 0.05 or less was considered statistically significant.

Figure 1. Overview of experimental protocol



A. Diagram of experimental setting with pig lying supine with site of occlusion highlighted. Location of microcirculation-, limb pressure and flow measurements shown in figure.



B. Experimental protocol over time (mins). Each small vertical line on the x-axis represents a measurement point. The arrow corresponds to t=0 i.e. the moment of initiation of therapy after which immediate measurement followed.
Abbreviations: B = Baseline, S = Stabilization period (10 minutes).
Symbol legend:
= creating stenosis, = thrombus induction, = release of the proximal clamp, = release of distal clamp

Results

Baseline and thrombus induction before start of therapy

Baseline parameters at the start of the experiments of all pigs and per subgroup are presented in Table I. Thrombus induction varied between pigs in duration and amounts of thrombin needed (Table II).

Table I. Baseline parameters

PARAMETER	TOTAL GROUP OF PIGS (n=10)	UROKINASE (n=4)	UROKINASE + BUBBLES (n=6)
Weight (kg)	60 (58-90)	60 (59-90)	61 (58-84)
MAP systemic (mmHg)	71 (51-100)	70 (59-98)	76 (51-100)
Systemic systolic pressure (mmHg)	94 (72-129)	94 (79-114)	93 (72-129)
Systemic diastolic pressure (mmHg)	62 (40-90)	58 (49-90)	68 (40-86)
Pulse frequency (pulses per minute)	64 (55-95)	64 (57-70)	64 (55-95)
Systemic T (degrees Celsius)	37.6 (35.5-38.5)	37.6 (37.0-38.2)	37.6 (35.3-38.5)
Flow in iliac artery (ml/min)	100 (73-289)	107 (74-250)	100 (73-289)
Microcirculation (PU)	44 (28-63)	38 (28-47)	51 (29-63)
T affected limb (degrees Celsius)	35.0 (31.2-37.0)	35.0 (34.3-35.7)	35.2 (31.2-37.0)
T control limb (degrees Celsius)	35.1 (28.3-36.4)	33.6 (33.3-35.6)	35.7 (28.3-36.4)
MAP affected limb (mmHg)	64 (45-83)	64 (45-83)	65 (53-80)
Systolic pressure affected limb (mmHg)	67 (50-95)	64 (50-93)	72 (64-95)
Diastolic pressure affected limb (mmHg)	57 (43-78)	57 (43-78)	60 (48-72)

Abbreviations: n= number, kg = kilograms, MAP= Mean Arterial Pressure, ml/min = milliliters per minute, mmHg = millimeter of mercury, PU = Perfusion Units, T = Temperature. Values presented are medians (range). Baseline parameters were measured after creation of the stenosis (50% flow reduction)

Table II. Thrombus-induction, changes in the limb with time and thrombus weight post-mortem

		UROKINASE					UROKINASE + BUBBLES						
		a1	a2	a3	a4	Median	b1	b2	b3	b4	b5	b6	Median
THROMBUS INDUCTION Duration (min)		150	110	90	152	130	190	120	177	110	160	97	140
Amounts of thrombin (U)		225	100	100	150	125	325	100	200	100	250	250	225
CHANGES IN	mL/min	3	0	0	0	0	121	90	98	0	0	16	53
Flow	%B	1	0	0	0	0	42	96	96	0	0	12	27
Microcirculation	PU	-19	-13	1	2	-6	9	13	38	0	-6	3	6
	%	-49	-52	5	12	-22	41	46	146	0	-24	6	23
MAP Limb	mmHg	-16	-4	-3	0	-3.5	33	38	54	11	2	-3	22
	%	-19	-16	-11	0	-13	55	52	104	41	9	-6	46
T affected limb	oC	0.3	-6.9	0.7	-1.5	-0.6	4.8	2.9	2.3	0.4	-5.2	-0.9	1.35
	%	1	-23	2	-6	-2	16	9	8	1	-17	-3	4
T control limb	oC	0.8	-0.9	-0.5	-3	-0.7	-0.9	-0.7	0.2	-0.7	-1	5.1	-0.7
	%	2	-3	-1	-9	-2	-3	-2	1	-2	-3	18	-2
THROMBUS WEIGHT	g	1.3	1.9	1.7	1.5	1.6	1.1	0.8	1.0	1.3	1.1	1.2	1.1

Change in various parameters due to therapy in individual pigs, i.e. t=180 vs. value after stabilization thrombus; designated in text as Δ. %B (B=Baseline) is defined as the following ratio: change in flow / baseline flow (flow after creating stenosis) *100%.

Abbreviations: ml/min = milliliters per minute, MAP = Mean Arterial Pressure, mmHg = millimeter of mercury, U = units, PU = Perfusion Units, T = Temperature

Changes during the experiment after start of therapy

Flow

After induction of the stenosis the median (baseline) flow was 100 ml/min (range 73-289). After execution of the occlusion protocol complete occlusion of the external iliac artery was reached in 7 out of 10 pigs. In three pigs some flow persisted, i.e. 29% (=84/289; UK+ group), 26% (=27/102; UK+ group) and 54% (=135/250; UK group) of their baseline flows.

After 3 hours of treatment, revascularization was regained in the UK+ group in 4 out of 6 pigs, with a median of 27% (range 0-96) of baseline flow and a mean increase of 61% (±42%). However, two of them already regained some flow during the stabilization period, as can be observed in Figure 2; right panel. Nevertheless, despite incomplete occlusion at the moment of initiation of therapy, arterial flow levels in these pigs showed marked increases during the rest of the procedure (up to 42% (=121/289) and 96% (=98/102) of baseline flow respectively). Of the 4 pigs with no arterial flow at the start of therapy, one (b2) showed higher flow, one showed temporary increase of flow (b6, reocclusion by embolization) while the other two (b4, b5) did not. Changes in flow are presented per pig in Table II. In the UK group, no increase in flow was observed in three out of 4 pigs within 3 hours of therapy (Figure 2; left panel). One pig had little increase in flow, although the occlusion of this pig's external iliac artery was partial at the time of release of the distal clamp (from 54% (=135/250) to 55% (=138/250) of baseline flow).

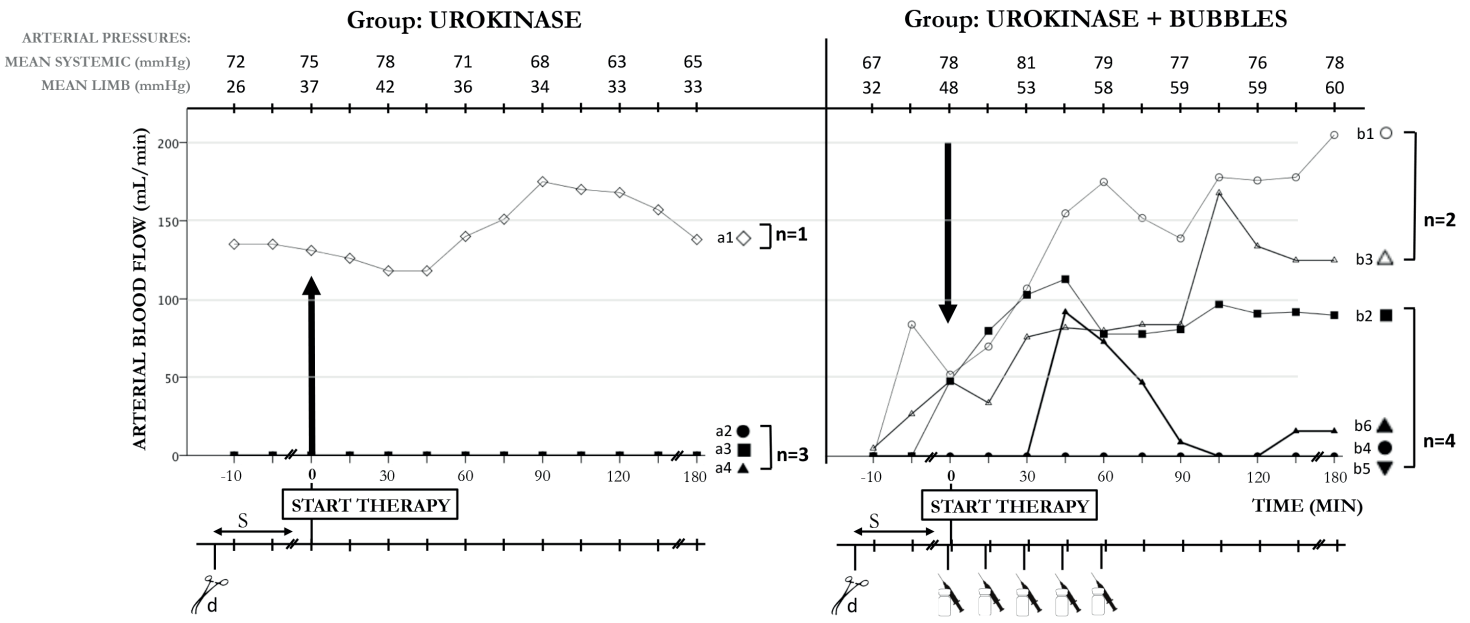


Figure 2. Arterial Blood Flow

Arterial blood flow of individual pigs. On top of the graph group mean systemic- and limb arterial pressures are shown. See table 2 for symbols corresponding to pig ID's. Unfilled symbols depict partial occlusion at the moment of initiation of therapy; filled symbols depict complete occlusion. The arrow corresponds to t=0 i.e. the moment of initiation of therapy after which immediate measurement followed. S = stabilization period.

= Administration of 1 vial of microbubbles, Baseline flow (flow after creating a stenosis) not shown here.

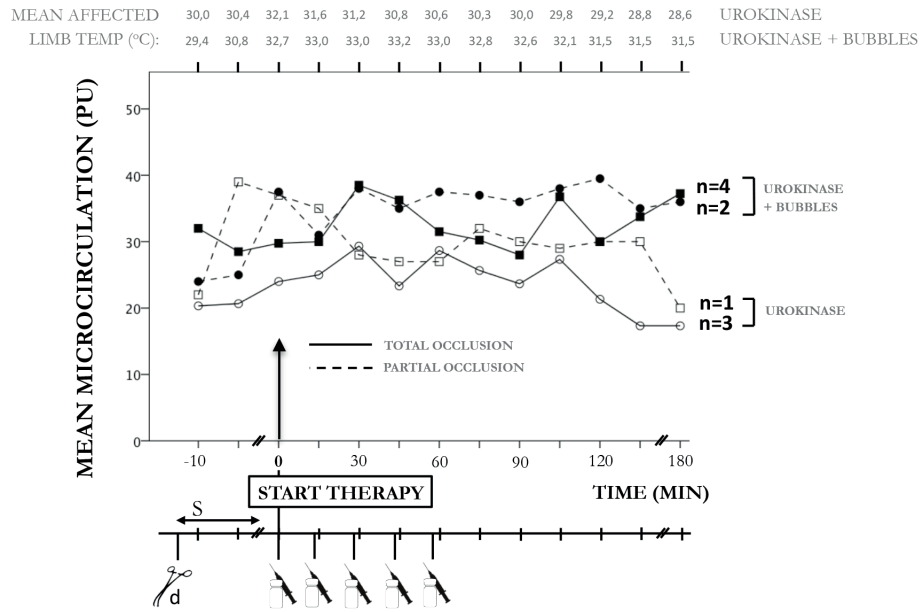


Figure 3. Microcirculation

Mean microcirculation curves. The two main groups, i.e. based on therapy, are each divided in 2 subgroups depending on whether there was total or partial occlusion at the moment of initiation of therapy. Unfilled symbols depict pigs treated with urokinase; filled symbols depict pigs treated with urokinase + bubbles. The large arrow corresponds to the moment of initiation of therapy. Continuous line depicts pigs with total occlusion, dashed line depicts pigs with partial occlusion at the moment of initiation of therapy.

Microcirculation

At the start of the experiments the median (baseline) microcirculatory flow was 44 Perfusion Units (PU, range 28-63). After execution of the occlusion protocol the median microcirculatory flow was 21 PU (range 11-31). The microcirculation partly remains because of the presence of existing collateral circulation.

In Figure 3 microcirculation levels are depicted for the different treatments and stratified for total- or partial occlusion at the moment of initiation of therapy. Microcirculation levels reflect a trend towards increase in pigs treated with urokinase + bubbles. Changes in microcirculation due to therapy (ΔMC, microcirculation at t=180 vs. microcirculation after stabilization thrombus) per pig are presented in Table II. They show an increase in microcirculation in 3 out of 6 pigs in the UK+ group, median change = 23% (-24 – 146), and only a slight increase

in 2 out of 4 pigs in the UK group (5% and 12%), median change = -22% (-52 – 12) in the whole UK group.

Pressures and temperature

Mean arterial limb pressures fluctuated amongst groups during the procedure showing a trend to increased pressures in the UK+ group (Figure 2, values at the top). Changes in mean arterial limb pressures (Δ limb pressure) per pig as presented in Table II show increased limb pressures in 4 out of 6 pigs in the UK+ group, median increase of 46% (-6–104), and decreased limb pressures in 3 out of 4 pigs in the UK group, median change in limb pressures of -13% (-19–0) in the whole UK group.

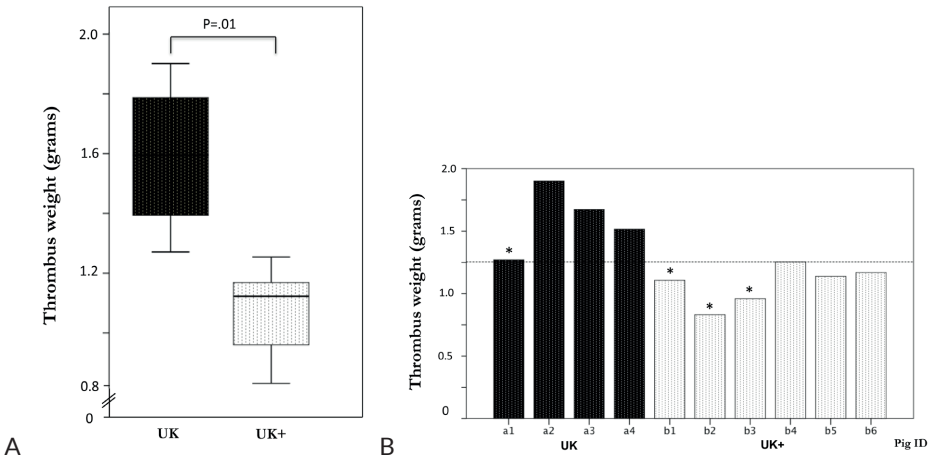
Systemic temperature of nearly all pigs (except b1) rose during the procedures (overall median increase in systemic temperature: 0.6°C (-0.1–2.2). There were slight temperature changes during the procedure in the affected limbs (Δ T affected limb) of all pigs (Table II), however none significant. Temperature of the control limbs (Δ T control limb) of all pigs remained the same (Table II).

In one animal of the UK+ group the pig was terminated before the experiment was ended after 135 minutes of therapy because of cardiac arrhythmias.

Thrombus weights and absence of bleeding complications at the end of the experiment

Thrombus weights were significantly lower in the pigs of the UK+ group when compared to the UK group, median 1.1g (0.8-1.3) vs. 1.6g (1.3-1.9) (P=.01; Figure 4). The weights of the 4 cm excised right external arteries varied with a maximum of 0.03g. Note that all the pigs in the UK+ group had lower thrombus weights post-mortem if compared to the pigs in the UK group; the exception in the latter group (pig a1) that showed already high flow at the moment of initiation of therapy. Importantly, no signs of hemorrhagic complications were observed during the procedures or in any of the organs investigated at autopsy.

Figure 4. Thrombus weights post-mortem



Thrombus weights post-mortem grouped and of all individual pigs. Asterisks mark pigs in which only partial occlusion was reached before initiation of therapy. NB: note the difference in y-axis between grouped (Figure 4A) and individual (Figure 4B) figures. Dark columns: UK group, light columns: UK+ group.

Discussion

In this study we investigated the therapeutic application of contrast-enhanced ultrasound with low-dose urokinase to enhance thrombolysis in order to lower the required doses of urokinase and to shorten therapy duration, both aiming at reducing complications. From a clinical perspective, thrombolytic therapy with urokinase for peripheral arterial occlusion takes one or more days at average to regain vascularization and ensure relief of symptoms[5]. In the present study we observed that in 3 hours of therapy-time, thrombus weights were on average 30% lower in animals treated with urokinase + bubbles. Furthermore we observed increases in arterial flow, microcirculation and limb arterial pressures in the experimental group, whereas in the UK group an increase in arterial flow was observed in only one out of four pigs and deterioration of microcirculation and limb arterial pressures. This raises the potential for contrast-enhanced ultrasound to enhance thrombolysis and shorten therapy duration in humans.

The most feared adverse events during thrombolytic therapy are bleeding complications, especially the occurrence of intracranial bleeding. During the performed procedures with low-dose urokinase, no signs of bleeding complications were observed. Although no conclusions regarding the incidence of (intracranial) bleedings can be drawn from this small number of subjects, it is likely that a lower

dose of urokinase lowers the risk of bleeding complications. Furthermore, shorter therapy duration could lower the risk of occurrence of other complications as well and most importantly lowers patient burden. In the present study the total therapy time was unfortunately limited to a maximum of 3 hours, due to ethical and practical reasons. However, in 3 hours marked improvement of reperfusion was observed in the group with microbubbles without any hemorrhagic complications.

In this study we used pigs for their resemblance to human cardiovascular anatomy and coagulation parameters. However, pigs are known to be allergic to nanoparticles' lipid shell, so we needed to provide medication to prevent allergic reactions[13,14]. To the best of our knowledge, no previous studies have been performed regarding thrombolysis with additional contrast-enhanced ultrasound in large animal models of peripheral arterial occlusion. In the setting of myocardial infarction Xie et al. showed improvement in epicardial recanalization rates as well as improvement in microvascular flow to the risk area with contrast-enhanced ultrasound and pro-urokinase-induced thrombolysis after acute coronary thrombotic occlusion in pigs[12]. This supports the results of our feasibility study. Importantly, treatment with contrast-enhanced ultrasound in addition to the administration of fibrinolytics could benefit the microcirculation as well. Clinically this could indicate potential improvement in the disease management of a patient's leg microcirculation. A potential explanation for the beneficial effects of contrast-enhanced ultrasound on the microcirculation could be a NO-dependent mechanism, as opted in the coronary setting[15].

The potential role of contrast-enhanced ultrasound in thrombolysis has also been shown in smaller animal models (rabbit iliofemoral arteries): Nishioka et al.[16] and Birnbaum et al.[17] showed dissolution of in-vivo thrombus after treatment with contrast-enhanced ultrasound solely, thus without the use of a thrombolytic drug. The (non-targeted) microbubbles were administered intra-arterially (Nishioka et al.) and intravenously (Birnbaum et al.), the latter importantly without loss of effectiveness compared to intra-arterial infusion. A drawback of treating thrombotic occlusions without fibrinolytics is the possible occurrence and persistence of distal emboli. The rabbit iliofemoral arteries treated with contrast-enhanced ultrasound showed no (0 out of 17, Nishioka et al.) or few cases (1 out of 10, Birnbaum et al.) of distal embolization. Distal emboli can occur during standard urokinase therapy, however, the continuous infusion of fibrinolytics dissolves them. In our study we also used continuous infusion of low-dose urokinase as part of the experimental treatment with microbubbles, which likely dissolves any distal emboli.

The therapeutic application of contrast-enhanced ultrasound and thrombolysis in humans is still in pilot stage. In a clinical pilot setting microbubbles in combination with ultrasound have shown a trend toward higher early recanalization and

clinical recovery rates in acute stroke patients when used as an adjunct to standard intravenous tPA therapy[9]. Despite limitations regarding sample size and deployment of operator-dependent techniques this trial shows promising results and the authors warrant continuation of clinical trials with contrast-enhanced thrombolysis. However, in the setting of acute stroke the safety in terms of intracranial hemorrhage and micro-embolization of this therapy needs to be established first, before evaluating its efficacy in a phase-II clinical trial setting. In the acute cardiac care setting a thrombolysis protocol with microbubbles was feasible and safe during treatment and follow-up[10]. In the latter, a trend toward a higher epicardial recanalization rate in patients treated with ultrasound and microbubbles seems present, although group sizes were too small in that pilot study to provide conclusions regarding patency rates. Patient inclusion of this trial is still ongoing. Contrast enhanced ultrasound combined with thrombolysis has not been applied in patients with critical limb ischemia.

For acute peripheral arterial occlusions in the limbs we recommend more studies on the optimization of contrast-enhanced ultrasound techniques for therapeutic thrombolytic use: different microbubbles and fibrinolytics as well as different infusion protocols could be used to enhance thrombolysis and minimize bleeding complications. In this study we were limited to our own low-dose urokinase thrombolysis protocol for translational purposes and we used a contrast agent that is clinically available and used in our current practice. However, utilization of other fibrinolytic agents such as tPA and other market-approved lipid-based contrast agents such as Definity (Lantheus Medical Imaging, MA, USA) have also proven feasible[9,11]. Furthermore we used freshly formed thrombus in our model, whilst the duration of 'acute' formed thrombus in patients at the moment of presentation with symptoms can vary from a few hours- to a few days. In addition we applied acute damage and induction of thrombotic occlusion in an otherwise 'normal' artery without atherosclerosis. Although we used a standardized thrombus induction protocol, in three out of 10 pigs limited flow persisted after release of the distal clamps. This variation can most likely be attributed to individual differences in coagulation profile. However, resembles the clinical situation of patients: a major reduction in flow only could lead to clinical symptoms and requirement of thrombolysis. Furthermore, changes in parameters due to therapy remain relevant (Table II).

Conclusion

In conclusion, we observed beneficial effects of contrast-enhanced ultrasound on thrombolysis with urokinase in extensive peripheral arterial occlusions: a significant reduction in thrombus weight was reached in the pigs receiving additional contrast-enhanced ultrasound. Moreover, iliac blood flow, microcirculation and limb arterial pressures tended to improve within 3 hours of therapy. Therefore, it

seems that time to thrombus resolution could be likely shortened. No hemorrhagic complications occurred during these experiments. Contrast-enhanced ultrasound has the potential to improve thrombolytic therapy in large peripheral arterial occlusions: this technique could result in faster revascularization and lowering of thrombolytic dose and therefore minimize complications. Our data warrant prospective studies in patients with peripheral arterial occlusions.

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Chapter 6: Intravenous targeted microbubbles carrying urokinase versus urokinase alone in acute peripheral arterial thrombosis in a porcine model.

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Abstract

Purpose

Standard therapy in acute peripheral arterial occlusion consists of intra-arterial catheter guided thrombolysis. As microbubbles may be used as a carrier for fibrinolytic agents and targeted to adhere to the thrombus, we can theoretically deliver the thrombolytic medication locally following simple *intravenous* injection. In this intervention-controlled feasibility study, we compared intravenously administered targeted microbubbles incorporating Urokinase and locally applied ultrasound, with intravenous Urokinase and ultrasound alone.

Materials and methods

In 9 pigs a thrombus was created in the left external iliac artery, after which animals were assigned to either receive targeted microbubbles and Urokinase (UK+tMB-group), or Urokinase alone (UK-group). In both groups, ultrasound was applied at the site of the occlusion. Blood flow through the iliac artery and microcirculation of the affected limb were monitored and the animals were euthanized one hour after treatment. Autopsy was performed to determine the weight of the thrombus and to check for adverse effects.

Results

In the UK+tMB-group (n=5), median improvement of arterial blood flow was 5ml/minute (range 0-216). Improvement was seen in 3 out of these 5 pigs at conclusion of the experiment. In the UK-group (n=4), median improvement of arterial blood flow was 0ml/minute (-10-18), with slight improvement in 1 out of 4 pigs. Thrombus weight was significantly lower in the UK+tMB-group (median 0.9383g (range 0.885-1.2809) versus 1.5399g (1.337-1.7628; P=0.017). No adverse effects were seen.

Conclusion

Based on this experiment, minimally invasive thrombolysis using intravenously administered targeted microbubbles carrying Urokinase combined with local application of ultrasound is feasible and might accelerate thrombolysis compared to treatment with Urokinase and ultrasound alone.

Key words

Targeted microbubbles, thrombolysis, arterial occlusion, ultrasound contrast agents

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Introduction

Acute peripheral arterial thrombosis is a condition threatening both limb and life. Treatment of acute peripheral arterial thrombosis with intra-arterial catheter guided thrombolysis is successful in many cases, reducing need for (redo) surgery[1,2]. However, thrombolysis takes time, while success of therapy greatly depends on duration of ischemia, i.e. time between onset of symptoms and reperfusion[3]. And, albeit minimal, intra-arterial thrombolysis remains invasive, as a catheter is needed for local administration of the fibrinolytic agent. Due to a possible longer duration of ischemia when using thrombolysis compared to surgical intervention, combined with the fact that the procedure still has an invasive aspect, further optimization of thrombolytic therapy is needed.

In patients with stroke as well as patients with coronary artery disease, clot lysis can be accelerated by local application of ultrasound (i.e. sonothrombolysis) and interesting results have been found in patients with peripheral arterial occlusive disease[4-6]. An even further acceleration of clot lysis can be achieved when ultrasound contrast agents (UCA's) are used[7-11]. In a previous study in a porcine model, we found promising results of combining intravenously administered microbubbles with standard intra-arterial catheter delivered Urokinase and locally applied ultrasound in acute peripheral arterial occlusion[12].

UCA's, or microbubbles, can be used as a vehicle for drug delivery: drugs can either be incorporated in the microbubble or attached to the outer layer[13-15]. Furthermore, by attaching Arg-Gly-Asp-Ser (RGDS), the recognition and binding site of platelet membrane glycoprotein 2b/3a receptor (GPIIb/IIIa), microbubbles can be targeted to adhere to the surface of a thrombus[16,17] (Figure I).

In combining incorporation of a fibrinolytic agent in targeted microbubbles (tMB) and destroying the tMB with high-intensity ultrasound once attached to a thrombus, we theoretically have a less invasive way of applying (local) intra-arterial thrombolysis, as placement of an intra-arterial catheter is no longer necessary. Hua et al showed promising results of targeted tPA loaded microbubbles in regards to efficacy and dosage of tPA needed in a rabbit model of small arterial occlusion. This model resembled small occluded arteries in ischemic stroke[18]. To objectively assess the feasibility of this less invasive treatment in large occluded arteries, as is the case in acute peripheral arterial occlusion in humans, we designed an intervention-controlled study using a porcine model. The hypothesis of the study was that systemically administered tMB with incorporated urokinase and locally applied ultrasound will enhance thrombolysis compared to systemic sonothrombolysis alone. To our knowledge, no previous studies have been published exploring the combination of intravenously administered, thrombus targeted and urokinase

loaded microbubbles with ultrasound therapy in an in-vivo model of acute peripheral arterial thrombosis.

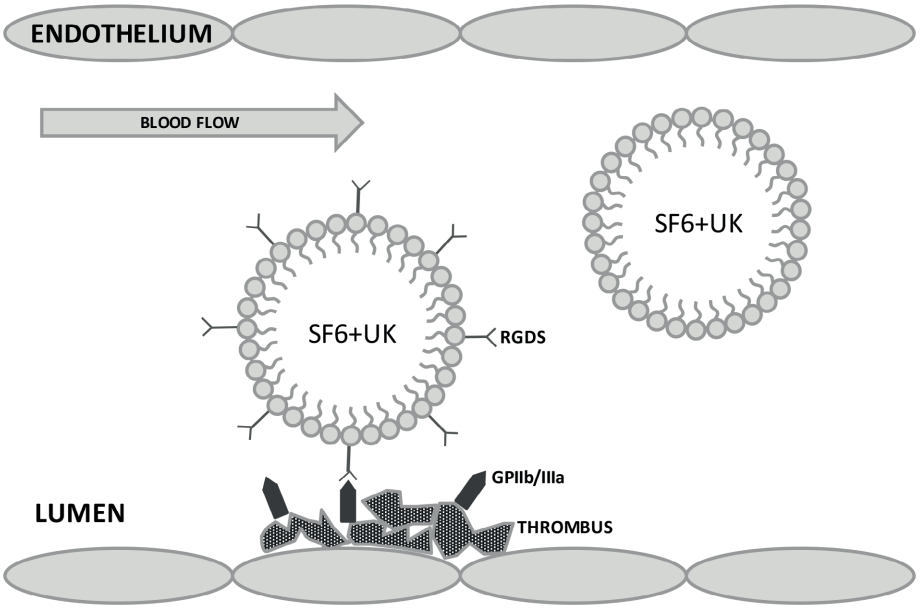


Figure 1. Schematics of targeted microbubble

SonoVue microbubbles which contain urokinase. The RGDS on the surface of the targeted microbubble binds to the glycoprotein 2b/3a receptor of the platelet membrane, keeping the microbubble at the site of the occlusion until it is destroyed with a high pressure ultrasonic wave and releases its contents locally.
SF6 = Sulphur hexafluoride, UK= Urokinase, RGDS = Arg-Gly-Asp-Ser, GPIIb/IIIa = glycoprotein 2b/3a receptor

Materials and methods

Approval of the Animal Ethics Committee was obtained before the start of the study and all procedures were done in accordance with the Dutch national guideline for humane animal treatment (Code Of Practice Welzijnsbewaking van proefdieren, 2004) as well as the European Directive 2010/63/EU on protection of animals used for scientific purposes.

Nine female Yorkshire pigs, 6 to 8 months old and with a median weight of 68kg (range 63-75), were housed at the research facility during one week before initiation of the protocol to allow for quarantine and acclimatization. Animals were

randomly assigned to either the intervention group: intravenously administered RGDS targeted microbubbles (tMB) combined with urokinase and local ultrasound (UK+tMB-group; n=5) or the control group: intravenously administered urokinase and local ultrasound (UK-group; n=4).

Before start of the procedure, all pigs received premedication with 40 milligrams (mg) of methylprednisolone and 500mg of indomethacin to prevent potential allergic reactions to the microbubbles. Pigs can be allergic to the lipids of the microbubble shell. Therefore, this protocol was developed in an earlier study, when 4 pigs showed severe allergic reactions to the SonoVue microbubbles[19]. In the same study, our protocol for inducing and maintaining anesthesia was described. In short, the pigs were sedated with an intramuscular injection of 28 mg per kg body weight ketamine (Alfasan, Woerden, The Netherlands), 0.5 mg per kg body weight midazolam (Actavis bv, Baarn, The Netherlands) and 1 mg of atropine (Pharmachemie, Haarlem, The Netherlands). Induction of anesthesia followed with an intravenous (i.v.) injection of 20 mg etomidate (B. Braun, Melsungen, Germany), followed by airway cannulation. During the procedure, we maintained anesthesia with endotracheally administered isoflurane, 1.5-2.0% (Pharmachemie, Haarlem, The Netherlands) and with 50 µg/h fentanyl (Hameln Pharmaceuticals, Hameln, Germany), 50 mg/h midazolam and 20 mg/h pancuronium (Organon, Oss, The Netherlands) intravenously. A catheter was placed in the right carotid artery to allow for regular arterial blood gas determination and to continuously monitor systemic blood pressure. Furthermore, heart rate, oxygenation and body temperature were measured during the entire procedure. Thermometers were placed between the toes of both hind legs and the left femoral artery was cannulated to monitor blood pressure of the affected limb. A laser Doppler probe (periflux 4001 master, Perimed, Järfälla, Sweden) was placed transcutaneously on the affected limb to measure microcirculation. As described previously[12,19,20], the left external iliac artery was identified via a midline laparotomy and the circumflex iliac artery was ligated. This to provide an adequate vessel length of at least 4 cm for our experiment. Blood flow in the iliac artery was measured using an ultrasonic flow probe (Transonic-Systems Inc, Ithaca, USA). A stenosis was created in the distal external iliac artery to more closely mimic a clinical situation, as well as to prevent dislocation of a fabricated clot. The diameter of the vessel was reduced with a vascular tourniquet, decreasing the flow in the iliac artery by 40-60%. To promote adhesion of thrombus to the vessel wall, the endothelium was damaged over four centimeters by clamping and declamping the iliac artery. The vessel was then clamped proximally and distally and 100 units of bovine thrombin (Calbiochem) were injected intraluminally. The proximal clamp was removed after 60 minutes, the distal clamp after 90 minutes. In case of persistent flow in the iliac artery, the vessel once again was occluded and another 100 units of thrombin were administered.

Once a thrombus was created, it was left to stabilize for another 10 minutes. After stabilization, an ultrasound probe (Philips Sonos 7500) with a diagnostic S3 transducer (Philips, Best, the Netherlands) was directed at the site of the thrombus in the iliac artery. In a human setting, the ultrasound probe would be placed on the skin directly over the target vessel. As closure of the abdominal wound would prevent adequate flow measurements through the iliac vessel, we simulated the clinical setting by placing a balloon filled with saline over the occlusion site. The ultrasound probe was placed on the balloon, resulting in a distance between the probe and the treatment vessel of 3cm. The mechanical index (MI) was set to 1.1 with a focus of 3cm and a frequency of 1.6 MHz.

The pigs in the UK+tMB-group (intervention group) now received an intravenous bolus injection of 500 000 units of urokinase combined with 2 vials of targeted microbubbles (10cc all together, containing 8µl of microbubbles per cc), followed by three repeated gifts of 50 000 units urokinase combined with 1 vial of targeted microbubbles every fifteen minutes (5cc), leading to a total treatment period of 1 hour. The ultrasound contrast agent used was SonoVue (Bracco Diagnostics Inc, Milan, Italy), which contains sulphur hexafluoride (SF6) encapsulated by phospholipids (Macrogol 4000, DSPC, DPPG, Palmitic acid). As described by Mu et al, RGDS adheres to components of the SonoVue microbubble outer layer via ionic bonds or physical adsorption[17]. To create the targeted microbubbles, we followed the same direct conjugation method, wherein the urokinase and RGDS peptide (Tocris bioscience, Bristol, UK) were added to 5ml of normal saline. The SonoVue powder was diffused in this solution and shaken for 1 minute to create the urokinase loaded targeted microbubbles. Before each injection, heart rate (BPM), blood pressure (mmHg) and temperature of the pig (°C) were measured, as well as blood flow through the vessel (ml/min), microcirculation in the affected limb (Perfusion Units; PU) and temperature of both limbs (°C). For the UK-group (control group), the same protocol was adhered to, with exclusion of the microbubbles. As UCA's are visible on ultrasound, the investigators were not blinded to the type of treatment given.

Ultrasound treatment was initiated during the start of infusion of the urokinase. In order for the microbubbles in the UK+tMB-group to satiate the treatment area, ultrasound impulses were applied intermittently (5 seconds off, 1 second on) until all microbubbles were destroyed. With a pulse duration of 3µs, the pulse repetition frequency was 24kHz, with a total exposure time of less than 10 minutes. One hour after last injection of urokinase, the pigs were euthanized and autopsy was performed. All organs were macroscopically inspected for possible (hemorrhagic) adverse events and tissue samples were taken. The weight of persisting thrombus in the iliac artery was measured. Primary endpoints were flow through the affected vessel and weight of thrombus at termination of the procedure. Secondary

endpoints were microcirculation, blood pressure and temperature of affected limb as well as any adverse events.

Data were analyzed with SPSS (IBM Statistics v20, Chicago, IL, USA). An unpaired Student's t-test or a Mann-Whitney-U test was used for comparison of continuous variables with (non-)parametric distribution and a Chi-square test was used to compare proportions between groups. A P-value of 0.05 or less was defined as statistically significant.

Results

Baseline characteristics were similar in both groups with regard to weight, core temperature, blood pressure both systemic and in the affected limb, flow through the iliac artery after creation of stenosis (= baseline flow) and percentage of flow reduction due to creation of the stenosis (Table I).

Median time to creation of thrombus was 170 minutes (range 93-200) in the UK+tMB-group versus 120 minutes (89-220) in the UK-group (P=0.524) and median units of thrombin needed for creation of the thrombus was 200 (range 100-225) in the UK+tMB-group and 163 (100-350) in the UK-group and (Table II).

Table I. Baseline parameters

PARAMETER	TOTAL GROUP OF PIGS (n=9)	UROKINASE + TARGETED MICRO-BUBBLES (n=5)	UROKINASE (n=4)	P
Weight (kg)	68 (63-75)	71 (63-75)	68 (66-73)	ns
MAP systemic (mmHg)	105 (65-118)	105(75-105)	98.5 (65-118)	ns
Systemic systolic pressure (mmHg)	123 (82-145)	123(106-126)	125 (82-145)	ns
Systemic diastolic pressure (mmHg)	95 (57-105)	95 (60-96)	85 (57-105)	ns
Heart rate (beats per minute)	78 (50-117)	75 (50-117)	80 (63-88)	ns
Systemic T (degrees Celsius)	37.2 (36.2-38)	36.8 (36.4-37.9)	37.6 (36.2-38)	ns
Flow in iliac artery (ml/min)	127 (104-213)	113 (104-207)	129 (111-213)	ns
Degree stenosis iliac artery (%)	46 (28-55)	45 (28-55)	51 (42-52)	ns
Microcirculation (PU)	36 (20-149)	39 (22-149)	31 (20-109)	ns
T affected limb (degrees Celsius)	34.5 (26.3-36)	34.2 (26.3-36)	34.9 (33.6-35.6)	ns
T control limb (degrees Celsius)	34.6 (30.1-35.3)	33.3 (30.1-34.6)	34.8 (34.6-35.3)	ns
MAP affected limb (mmHg)	74 (55-108)	70 (60-108)	78 (55-95)	ns
Systolic pressure affected limb (mmHg)	103 (70-133)	101 (74-133)	103 (70-124)	ns
Diastolic pressure affected limb (mmHg)	68 (47-97)	68 (53-97)	65.5 (47-81)	ns

Abbreviations: n= number, kg = kilograms, MAP= Mean Arterial Pressure, ml/min = milliliters per minute, mmHg = millimeter of mercury, PU = Perfusion Units, T = Temperature, ns = not significant. Values presented are medians (range)

Table II. Thrombus-induction, changes in the limb with time and thrombus weight post-mortem.

		UROKINASE + TARGETED MICROBUBBLES						UROKINASE					
		a1	a2	a3	a4	a5	Median	b1	b2	b3	b4	Median	P
THROMBUS INDUCTION Duration (min)		93	98	200	170	173	170	120	89	120	220	120	ns
Amounts of thrombin (U)		100	100	200	200	225	200	100	100	225	350	163	ns
CHANGES IN Flow	ml/min	216	97	0	5	0	5	-10	0	18	0	0	ns
	%B	104	86	0	5	0	5	-8	0	6	0	0	ns
Microcirculation	PU	29	139	-13	7	11	11	2	-2	-4	3	0	ns
	%B	42	93	-46	18	50	42	2	-10	-16	8	-4	ns
MAP Limb	mmHg	30	-17	-13	13	-16	-13	11	-6	6	-13	0	ns
	%B	42	-35	-20	17	-31	-20	14	-14	11	-20	-1.5	ns
T affected limb	oC	4.3	7.3	-4.9	1.8	-4.5	1.8	-0.7	1	1.4	-0.8	0.15	ns
	%B	12	28	-17	5	-13	5	-2	3	4	-2	0.5	ns
T control limb	oC	1.4	4	-0.4	0.4	-0.3	0.4	-0.9	-0.2	0.3	3.6	0.05	ns
	%B	4	13	-1	1	-1	1	-3	-1	1	10	0	ns
THROMBUS WEIGHT	g	0.9	0.9	1.0	0.9	1.3	0.9	1.3	1.5	1.6	1.8	1.5	0.017

Change in various parameters due to therapy in individual pigs, i.e. t=135 vs. value after stabilization thrombus; designated in text as Δ.
%B (B=Baseline) is defined as the following ratio: change in flow / baseline flow*100%
Abbreviations: ml/min = milliliters per minute, MAP = Mean Arterial Pressure, mmHg = millimeter of mercury, U = units, PU = Perfusion Units, T = Temperature, ns = not significant

Arterial blood flow

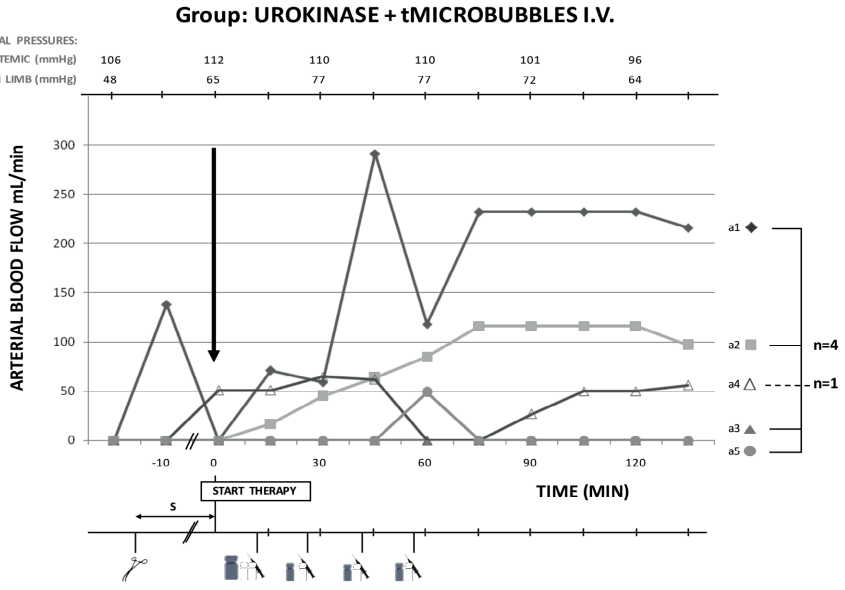
A complete occlusion was reached in 6 out of 9 pigs. There was some residual flow through the iliac artery in 3 pigs, respectively 49% (51/104ml/min; UK+tMB-group), 38% (=50/131ml/min; UK-group) and 4% (9/213ml/min; UK-group) of baseline flow.

In the UK+tMB-group, 4 out of 5 pigs showed increase in arterial blood flow during the experiment. One pig showed a complete return to baseline flow (216/207ml/min =104%) at the end of the procedure. Of the other 4 pigs in the UK+tMB-group, 2 more showed improvement in arterial flow at conclusion of the experiment. One of these had a partial occlusion at start of therapy and showed an increase in arterial flow of 5ml/min. The second had complete occlusion at initiation of therapy and showed an increase of 97ml/min, returning to 86% of baseline flow. Of the last 2 pigs in the UK+tMB-group, 1 showed a temporary increase in flow (49/130ml/min = 38% of baseline flow) followed by re-occlusion, most likely due to peripheral embolization (Figure 2a).

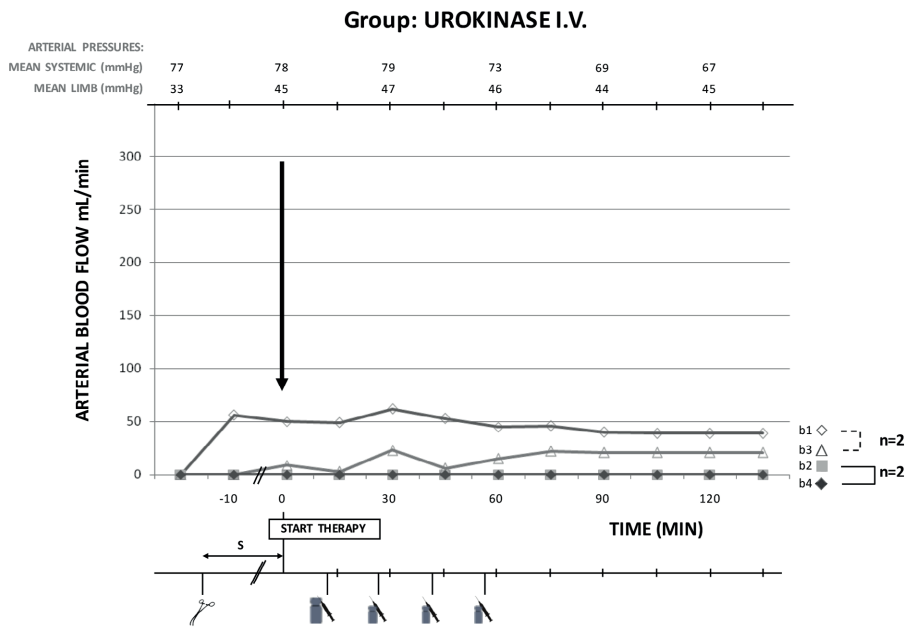
In the UK-group, 2 out of 4 pigs had a partial occlusion at start of therapy. One of these showed slight improvement of arterial flow at the end of the experiment (18ml/min) and one showed a decrease in blood flow of 10ml/min; the 2 pigs with a complete occlusion at the start of treatment did not show any improvement with UK therapy (Figure 2b).

Figure 2. Arterial blood flow

A



B



Arterial blood flow of individual pigs in the intervention group (**Figure 2a**) versus the control group (**Figure 2b**). Group mean systemic- and limb arterial pressures are shown at the top of both graphs. Unfilled symbols depict partial occlusion at start of therapy; filled symbols depict complete occlusion. The arrow corresponds to the moment of initiation of therapy, i.e. t=0. S = thrombus stabilization period.

= Administration of: = 500 000U Urokinase, = 50 000U Urokinase, = 1 vial of microbubbles

Microcirculation

Median baseline microcirculatory flow was 36 PU (range 20-149), 39 in the UK+tMB-group and 32 in the UK-group (Table I). After stabilization of thrombus, median microcirculation in the entire group decreased to 27 PU (range 9-35). In Table II, differences in microcirculation at initiation of therapy versus at the end of the experiment are shown per individual pig. Out of the 5 pigs in the UK+tMB-group, 4 showed increase in microcirculation (range -13-139 PU); median difference 43%. In the UK-group, 2 out of 4 pigs show a slight increase in microcirculation (range -4-3 PU) with a median difference of -4%. In figure III, mean microcirculation curves of the 2 treatment groups are shown, divided in 2 subgroups depending on complete or partial occlusion at start of therapy (Figure 3).

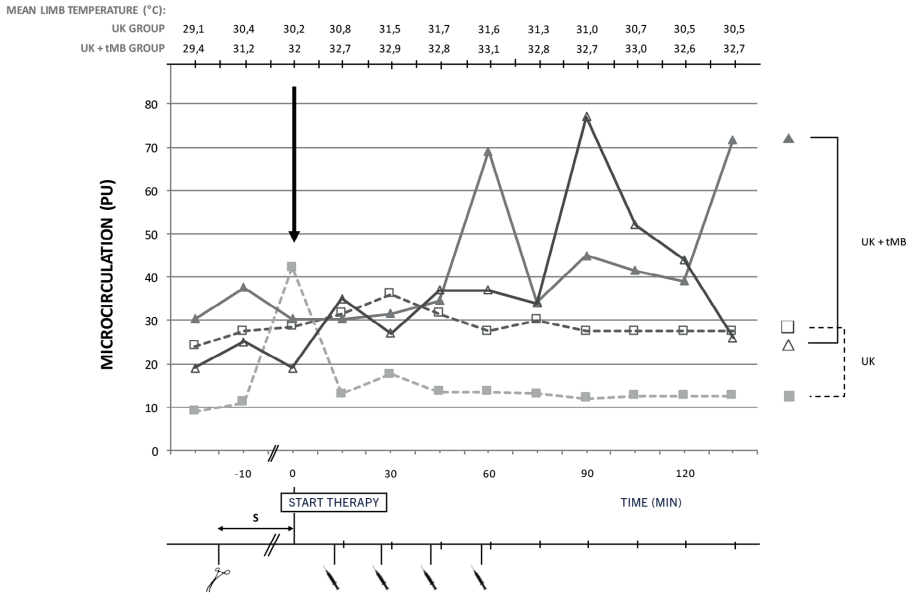


Figure 3. Microcirculation

Mean microcirculation curves of the two groups, divided in 2 subgroups depending on total or partial occlusion at the start of therapy. Unfilled symbols depict partial occlusion at start of therapy; filled symbols depict complete occlusion. The arrow corresponds to the moment of initiation of therapy, i.e. t=0. S = thrombus stabilization period.

= Administration of UK with or without microbubbles

Blood pressure and temperature

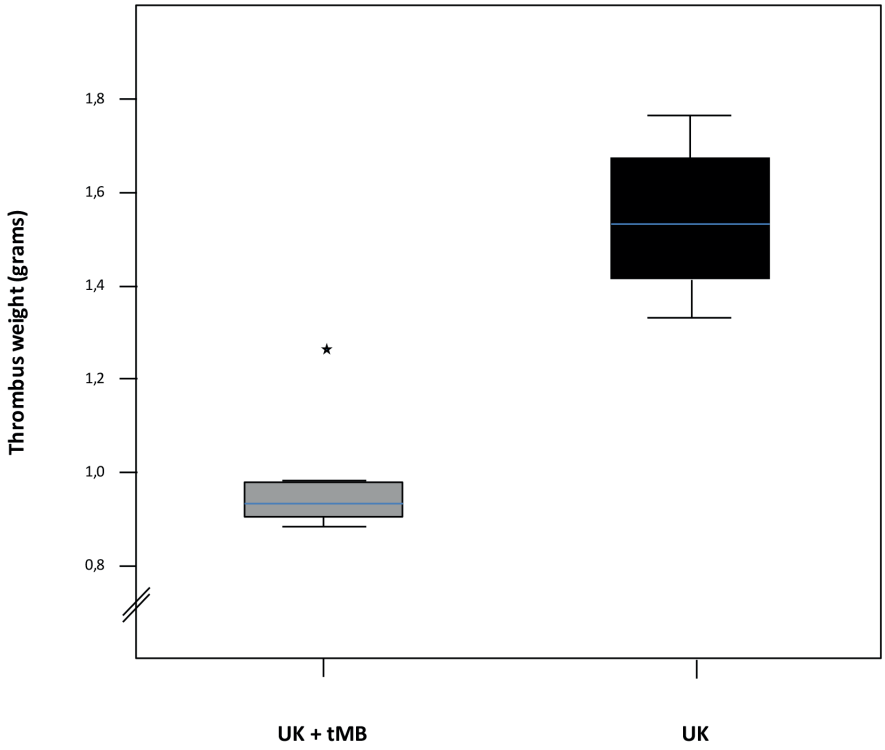
Mean systemic arterial pressures dropped 15% in both groups during the experiment. In both the UK+tMB-group and UK-group mean limb arterial pressures varied only slightly. There was no significant difference between mean limb arterial pressures at the start of therapy versus at the end of the experiment (ΔMAP limb) in either group, nor was there a difference noticeable between the groups (Table II/Figure 2).

During the procedure, systemic temperatures of all but 1 pig (b1) rose, with a median increase of 1.2°C (range -0.7-2.9). All pigs showed slight temperature changes in the affected limb from baseline temperature to end of procedure (ΔT affected limb), but there were no significant differences between both groups. The same was true for temperatures of the control limb (Table II).

Thrombus weight

The median thrombus weight at the end of the procedure was 0.9383g (0.885-1.2809) in the UK+tMB-group and 1.5399g (1.337-1.7628) in the UK-group (P=0.017) (Figure 4). All pigs in the UK+tMB-group had a lower thrombus weight at autopsy than pigs in the UK-group. In the UK+tMB-group, thrombus weighed between 0.89 and 0.98g in 4 out of 5 pigs. The last pig in the UK+tMB-group (a5) however, had a markedly higher thrombus weight of 1.28g.

Figure 4. Thrombus weight post-mortem
A



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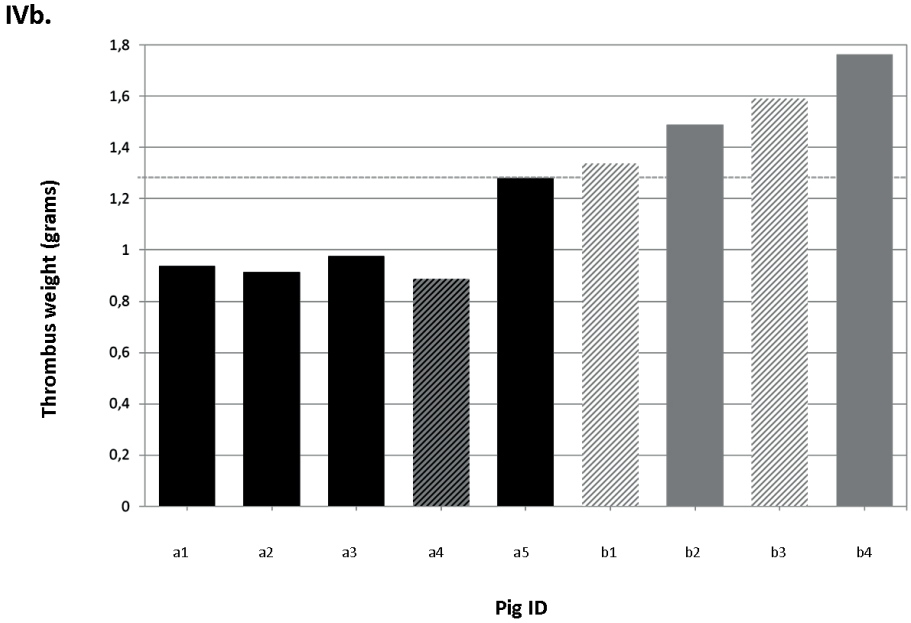

Thrombus weights post-mortem grouped (a) and of individual pigs (b). NB: there is a difference in y-axis between grouped and individual figures.
UK = Urokinase, tMB = targeted Microbubbles

UK + tMB group, complete occlusion

UK + tMB group, partial occlusion at start of therapy

UK group complete occlusion

UK group, partial occlusion at start of therapy

Adverse events

Apart from possible embolization in 1 pig (a5), no adverse events were encountered during the experiments and no signs of adverse effects were found at autopsy, especially no signs of hemorrhage.

Discussion

Outcome of acute peripheral arterial thrombosis varies from complete recovery to amputation and greatly depends on duration of ischemia[3]. Optimization of therapy is needed to increase limb survival. The combination of fibrinolytic therapy, ultrasound and UCA's (ultrasound contrast enhanced sonothrombolysis) shows promising results regarding time to reperfusion in both cerebral and myocardial

infarction[4,9,21], as well as in our earlier study in a porcine model of acute peripheral arterial occlusion[12]. Further optimization of thrombolysis might be achieved by avoiding use of intra-arterial catheters and by accelerating lysis.

UCA's can be loaded with a drug and targeted to adhere to a specific site. Once attached to this site, they can be destroyed with high-intensity ultrasound, releasing the preloaded drug at the designated location[14]. In this study, we used UCA's as a vehicle for the fibrinolytic agent, thus avoiding placement of an intra-arterial catheter. The use of the carrier function of thrombus targeted microbubbles to create a form of minimally invasive local intra-arterial thrombolysis has -to our knowledge- not been described before in an *in vivo* model of acute peripheral arterial occlusion.

At the end of this short experiment, we observed an increase in arterial blood flow in 3 out of 5 pigs in the UK+tMB-group, whereas only 1 out of 4 pigs in the UK-group had a slight increase in arterial blood flow. Only partial occlusion was reached in 3 out of 9 pigs at start of therapy. Two of the pigs with only partial occlusion at start of therapy were treated in the UK-group. One of these showed further deterioration during the experiment and the other showed only minor improvement. Therefore, reaching only partial occlusion in 3 out of the 9 pigs does not bias our experiment in favor of the UK+tMB-group. Thrombus weights at autopsy were significantly lower in the UK+tMB-group versus the UK-group. Interestingly, one pig in the UK+tMB-group (a5) had a markedly higher thrombus weight of 1,28g compared to other pigs in this group. This was the same pig that had a temporary increase in arterial blood flow during the experiment, after which the iliac artery re-occluded. The temporary increase in blood flow as well as the higher thrombus weight might be explained by embolization occurring during therapy. We did not find sufficient evidence to prove or disprove this theory at autopsy. Apart from possible distal embolization in this one case, there were no signs of adverse events during the experiments or at autopsy. Based on this experiment, a less invasive manner of sonothrombolysis seems feasible for acute peripheral arterial occlusion.

We realize there are some limitations to this study. Duration of therapy was shorter than is usual in a clinical setting and we did not examine possible long term effects of the tMB combined with urokinase and ultrasound on local tissue. However, based on ethical considerations of animal welfare, longer duration of the experiment was not justified. Study population was kept small based on the same ethical principles.

Although some promising results were found in this experiment, further research on biomechanical properties of the microbubbles is necessary. More information is needed towards the exact concentration of incorporated Urokinase in the microbubble , the concentration of RGDS on the outer layer of the microbubble

and the therapeutic effect of the RGDS, before this therapy can be investigated in patients. We will conduct *in-vitro* studies and investigate the targeted microbubbles on their stability, dose response- and temporal efficacy in order to assess their optimal configuration.

Conclusion

This study showed that minimal invasive thrombolysis is feasible using intravenously administered targeted microbubbles carrying urokinase combined with local application of ultrasound. This technique is effective and might accelerate thrombolysis, which is potentially beneficial in patients with acute peripheral arterial thrombosis. Further research regarding stability, dose response- and temporal efficacy is needed to assess optimal configuration of the microbubbles.

Acknowledgements

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Chapter 7: Feasibility of microbubble accelerated low-dose thrombolysis of peripheral arterial occlusions using an ultrasound catheter

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Abstract

Purpose
To evaluate the feasibility of combining microbubble infusion with ultrasound catheter directed thrombolysis for peripheral arterial occlusions.

Methods
The effects of the ultrasound (US) catheter (EkoSonic Endovascular Device) on microbubble survivability were investigated in an in-vitro model in which the ultrasound catheter was placed into a vascular phantom. Microbubble infusion was done either through the drug delivery lumen of the ultrasound catheter (DDC, n=10 without US, n=10 with US) or through the lumen of the vascular phantom (intravascular infusion, n=10 without US, n=10 with US). Microbubble survivability was assessed by measuring bubble samples pre- and post-infusion and corrected for dilution. Two groups (n=5 per group) were defined for each of the infusion methods: the ultrasound catheter was either OFF or continuously ON.

In a second experiment, the thrombolytic efficacy was investigated in-vivo in a porcine model (n=5) with a 4 cm peripheral arterial occlusion. The treatment consisted of microbubble infusion through the drug delivery lumen of the ultrasound catheter during the first hour of continuously ON ultrasound assisted thrombolysis with a low-dose urokinase protocol (50.000 IU/hour) for 3 hours. The measured determinants for lytic effect were thrombus weight and remaining length of thrombus post-mortem.

Results
The ultrasound setting significantly influenced microbubble survivability between the OFF vs. ON groups (P<.001) with a relative decrease in concentration of 42% when the ultrasound setting was ON. Microbubble survivability was not significantly influenced by mode of infusion through the DDC or tube lumen of the vascular phantom. In the porcine model, the median thrombus weight post-mortem was 1.02 grams (0.96-1.43). The median length of the remaining thrombus after 3 hours of treatment was 2.25 cm (1.5-4.0). These are significantly lower median thrombus weight and shorter median thrombus length as compared to our historic control group.

Conclusion
Microbubbles can be combined with an ultrasound catheter and could potentially accelerate thrombolytic treatment of peripheral arterial occlusions. Future studies are warranted to investigate how best to combine the microbubbles with the ultrasound catheter before clinical application.

Introduction

Catheter directed thrombolysis (CDT) has been an effective treatment for patients with non-threatening acute limb ischemia for the last two decades.[1] Limitations of this treatment include bleeding complications in up to 13% of patients and an extended time needed for revascularization, which places a heavy burden on the patient.[2,3] Therefore, acceleration of thrombolytic treatment is needed to minimize risks and limit patient burden.

Recently, several studies have investigated ultrasound (US) as an accelerator of thrombolytic treatment for peripheral arterial occlusions.[4-7] The potential effects of ultrasound on clot dissolution include mechanical and chemical effects such as acoustic cavitation, microstreaming, mechanical erosion, intracellular microcurrents, thermal warming and increased clot permeability. The only randomized controlled clinical trial (RCT) comparing ultrasound accelerated thrombolysis (n=18) to conventional catheter directed thrombolysis (n=32) showed that therapy time for thrombolytic treatment is significantly reduced with ultrasound accelerated thrombolysis vs. conventional thrombolysis (17.7 vs. 29.5 hours, respectively, P=.009).[8] However, this decrease came at the cost of major bleeding complications in both treatment arms (11 vs. 6%, respectively) including 2 cases of intracranial bleedings. Potentially this is due to the high dose of fibrinolytic agents administered (100,000 International Unit per hour), since several RCTs from the 1990s report similar bleeding rates with a high dose protocol.9,10

Another technique to potentially accelerate thrombolysis is contrast-enhanced ultrasound, utilizing micro sized gas-filled bubbles (i.e. microbubbles (MBs)) that collapse when exposed to high-intensity US and augment the effects of ultrasound on clot dissolution.[11] This technique has been evaluated in a porcine model of peripheral arterial occlusion and accelerated catheter-directed thrombolysis with a low-dose urokinase protocol.[12] Limitations include the external application of US, which has inter-operator variability and the safety, feasibility and application of this technique has yet to be investigated in clinical studies. In addition, the administration of microbubbles in this technique is intravenous, whereas intra-arterial administration of microbubbles theoretically could result in a higher local dosage of microbubbles and therefore more clot dissolution effect.

A treatment using an ultrasound catheter in combination with intra-arterial infusion of microbubbles through its drug delivery lumen could increase efficacy of thrombolysis without inter-operator variability of ultrasound application. This concept has recently been illustrated with in-vitro data combined with an in-vivo model of inferior vena cava thrombosis treated with a custom-designed ultrasound catheter and lab produced microbubbles.[13] However, no data is currently available

for models of peripheral arterial occlusion and furthermore the effects of clinically available ultrasound catheters on FDA/EMEA allowed microbubbles are as yet unknown.

The aim in the present paper is twofold: to assess whether microbubbles can be used in combination with an ultrasound catheter, and to assess the lytic efficacy of microbubble accelerated low-dose thrombolysis using an ultrasound catheter in a validated porcine model of peripheral arterial occlusion.[14]

Methods

In-vitro model

In an *in-vitro* set-up (Figure 1) an ultrasound catheter (EKOS EkoSonic System, EKOS Corp, Bothell WA, USA,) was placed into a vascular phantom.[15] Two infusion pumps (Perfusor FM, B. Braun, the Netherlands) were attached to the catheter system to infuse saline at a rate of 0.3 mL/min through the drug delivery lumen of the catheter (DDC) and at a rate of 50 mL/hour through the coolant lumen. A third pump infused saline into the tube lumen of the vascular phantom to mimic blood flow at a rate of 1 mL/min. All pumps were validated for exact flow rates to correct small deviations in flow.

SonoVue microbubbles (Bracco International B.V., Amsterdam, the Netherlands, clinically approved for diagnostic use in Europe) were 1000x or 100x diluted with saline to a concentration in the range of 10^7/ml or 10^8/ml and used to replace the saline infusion through the drug delivery lumen of the catheter or through the vascular phantom lumen.

The pumps ran for 5 minutes before the start of the experiments to fill the dead spaces in the in-vitro setup. Immediately after the 5 minutes of priming the pumps ran for 10 minutes per experiment at constant flow rates. The part of the vascular phantom which contained the catheter treatment area was submerged in a 37˚ Celsius water bath. The mix of drug delivery flow and vascular phantom flow was collected in a glass column. The saline that was infused through the coolant lumen, was collected separately.

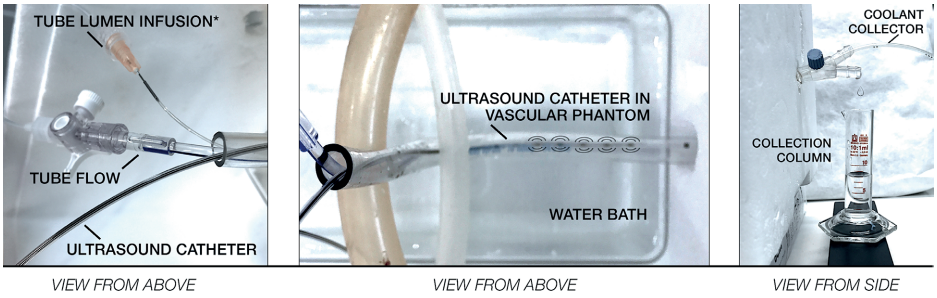


Figure 1: in-vitro set-up with ultrasound catheter in vascular phantom

Bubble survivability

Bubble survivability of the SonoVue microbubbles was measured by comparing microbubble concentrations pre- and post-infusion through the catheter system. For the microbubble concentration pre-infusion an average of two samples at t=0 min and t=10 min was used to correct for differences through time. The microbubble concentration post-infusion was corrected for dilution by tube flow based on the total infused- and collected volumes. Microbubble samples were analyzed using a MultiSizer 3 Coulter counter (Beckman Coulter Nederland B.V., the Netherlands) using a 50 um aperture (detection limits 1.0-30.0 um) and 0.9% saline buffer (100 uL sample volume, 100 mL electrolyte volume, 500 uL analytic volume). Different groups (n=5 per group) were evaluated based on the ultrasound catheter settings, i.e. either OFF or continuously ON.

Microbubble infusion was either through the drug delivery lumen of the ultrasound catheter (DDC) to simulate intra-arterial infusion nearby the thrombus, or into the tube lumen of the vascular phantom directly proximal to the catheter treatment area, to simulate systemic intravenous microbubble infusion.

Porcine model of peripheral arterial occlusion

The lytic efficacy was evaluated in a porcine model (n=5) of peripheral arterial occlusion. Approval of the Animal Ethics Committee (AEC) was obtained before initiation of the study (local registration number FYS 10-11). Five Yorkshire pigs with a median weight of 67 kg (64-74) were used. The porcine model of peripheral arterial occlusion was used as previously described (Lab Animal 2014). After creation and stabilization of a 4cm thrombus in the external iliac artery, this experimental treatment group was treated for 3 hours with an ultrasound catheter (continuously ON) and 4 vials of SonoVue microbubbles infused through the DDC during the first hour. A low-dose thrombolysis protocol was used with 50,000 International Units of urokinase (Medac GmbH, Hamburg, Germany) per hour.

The measured determinants for lytic effect were thrombus weight and remaining length of thrombus post-mortem.

Statistics

Data was analyzed using SPSS (IBM Statistics v20, Chicago, IL, USA and presented as medians (range) in case of non-parametric distribution or as means ±SD in case of a parametric distribution. A Mann Whitney-U test was used to compare continuous variables with non-parametric distributions between groups. A bonferroni correction was used in the case of multiple comparisons to decrease the likelihood of a Type I error. A P value less than .05 was considered statistically significant.

Results

Microbubble survivability

Data from the experiments are presented in Table I, included are pre- and post-infusion microbubble characteristics and survivability.

Table I: Microbubble survivability results of the different experimental groups

A: 50um aperture used, n=5 each group

			US OFF			US ON		
			pre	post	Δ% %Survival	pre	post	Δ% %Survival
DDC	MB diam (range)	um	1.56 (1.47-1.65)	1.69 (1.55-1.83)	6.5 (3.6-16.2)	1.62 (1.50-1.69)	1.46 (1.43-1.50)	-7.9 (-13.9;-0.2)
	MB conc (range)	*10^7/mL	1.13 (0.85-1.36)	0.94 (0.84-1.45)	94 (70-99), 100	1.35 (1.00-1.90)	0.78 (0.60-1.02)	58 (37-81)
TUBE LUMEN	MB diam (range)	um	1.58 (1.52-1.86)	1.61 (1.49-1.69)	3.8 (-2.1;5.5)	1.61 (1.56-1.88)	1.46 (1.41-1.48)	-9.4 (-21.2;-6.1)
	MB conc (range)	*10^7/mL	1.25 (1.07-1.36)	1.01 (0.81-1.14)	81 (72-91)	1.38 (1.09-1.68)	0.59 (0.55-0.66)	46 (35-60)

B: 30um aperture used, n=5 each group

			US OFF			US ON		
			pre	post	Δ%	pre	post	Δ%
DDC	MB diam (range)	um	0.99 (0.95-1.14)	0.91 (0.85-0.97)	-10.2 (-20.0; 3.79)	1.16 (1.15-1.22)	0.90 (0.89-0.91)	-22.5 (-25.6;-21.2)
	MB conc (range)	*10^7/mL	7.44 (6.48-10.21)	9.16 (4.95-9.79)	81 (69-99)	7.05 (5.89-10.05)	5.48 (5.22-5.65)	68 (49-70)
TUBE LUMEN	MB diam (range)	um	1.09 (1.05-1.15)	1.05 (0.91-1.06)	-6.3 (-14.0; -1.4)	1.12 (1.10-1.18)	0.90 (0.89-0.91)	-19.7 (-19.0;-22.5)
	MB conc (range)	*10^6/mL	9.48 (8.18-10.67)	9.09 (7.04-9.20)	73 (55-93)	8.95 (7.74-10.54)	8.71 (8.20-10.28)	42 (28-47)

Presented are median pre- and post-infusion diameters and concentrations of the experimental groups with either infusion through the Drug Delivery lumen of the Catheter (DDC, n=10) or tube lumen (n=10) with the UltraSound (US) core either switched OFF (n=10) or ON (n=10). Survivability is presented based on the pre- and post-infusion concentrations of samples corrected for dilution based on the total infused and collected volumes.
Abbreviations: DDC = Drug Delivery lumen Catheter, US = ultrasound, MB = microbubble, diam = diameter, conc = concentration, **Δ% =** relative difference post vs. pre-infusion MB diam expressed in %, %survival = MB post-conc/MB pre-conc expressed in %.

Mode of infusion: DDC vs. tube lumen

The mode of infusion of microbubbles through the DDC (simulating intra-arterial infusion) or into the tube lumen of the vascular phantom (simulating systemic intravenous infusion) did not significantly influence the bubble survivability. This is illustrated by the small differences between the DDC (blue) and tube lumen (red) bars within the two US setting groups (Figure 2). Nor did it significantly influence the size of the microbubbles.

Ultrasound setting: OFF vs. ON

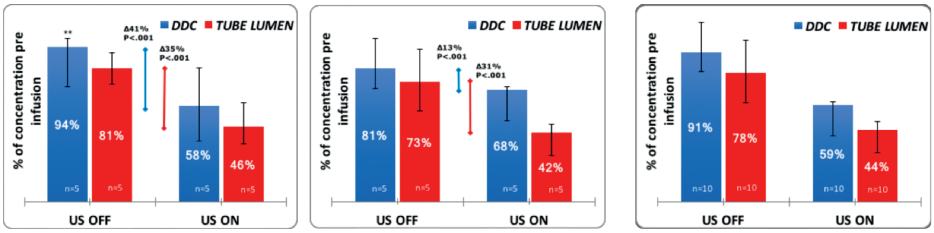
Whether the ultrasound was OFF or ON influenced microbubble stability significantly. For DDC infusion with ultrasound OFF (50um aperture, 1000x dilution), a median 94% (70-99) of the microbubble concentration was measured post infusion, decreasing to a median 58% (37-81) in the ON group, (P<.001). For the 30um aperture measurements with 100x dilution a median 81% (69-99) of the microbubble concentration was measured post infusion, decreasing to a median 68% (49-70) in the ON group, (P<0.001).

For tube lumen infusion with ultrasound OFF (50um aperture, 1000x dilution), a median 81% (72-91) of the microbubble concentration was measured post infusion, decreasing to a median 46% (35-60) in the ON group, (P<.001). For the 30um aperture with 100x dilution a median 73% (55-93) of the microbubble concentration was measured post infusion, decreasing to a median 42% (28-47) in the ON group, (P<.001). This illustrates a relative decrease in concentration of 41% and 35% (50um aperture, 1000x dilution) and 13% and 31% (30um aperture, 100x dilution) when the ultrasound setting is ON in the DDC- and tube lumen infusion groups, respectively.

In addition, ultrasound application influenced the median size of the microbubbles significantly. For DDC infusion with ultrasound OFF (50um aperture, 1000x dilution), a median change in size of the microbubbles of +6.5% (3.6-16.2) was measured between pre- and post-infusion, vs. a change of -7.9% (-13.9;-0.2) with ultrasound ON, (P=.014). For the 30um aperture with 100x dilution a median change in size of the microbubbles of -10.2% (-20.0;3.79) was measured between pre- and post-infusion with ultrasound OFF, vs. a change of -22.5% (-25.6;-21.2) with ultrasound ON, (P=.008).

For tube lumen infusion with ultrasound OFF (50um aperture, 1000x dilution), a median change in size of the microbubbles of +3.8% (-2.1;5.5) was measured between pre-and post-infusion, vs. a change of -9.4% (-21.2;-6.1) with ultrasound ON, (P=.014). For the 30um aperture with 100x dilution a median change in size of the microbubbles of -6.3% (-14.0;-1.4) was measured between pre-and post-infusion, vs. a change of -19.7% (-19.0;-22.5) with ultrasound ON, (P=.008).

Figure 2: Microbubble survivability after infusion in a vascular phantom, 50um aperture (2A), 30um aperture (2B) and all experiments combined (2C)



Depicted are medians (range) of groups with n=5. Microbubbles were infused either through the drug delivery lumen of the catheter (DDC, simulating intra-arterial infusion) or directly into the vascular phantom (simulating intravenous infusion). Bubble survivability is measured by the microbubble concentration ratio post/pre infusion. The post infusion concentration was corrected for dilution by the tube flow following from the infusion rates and collected volumes.

Abbreviations: DDC = Drug Delivery lumen Catheter, US = ultrasound n = amount of experiments, **Δ**=relative difference between groups, ** = 2 outliers (100, 100).

Lytic efficacy

In a series of different experiments in a porcine model as described in the methods section of this manuscript, the lytic efficacy was evaluated. The median thrombus weight post-mortem was 1.02 grams (0.96-1.43). The median length of the remaining thrombus after 3 hours of treatment was 2.3 cm (1.5-4.0). In previous experiments of conventional catheter-directed thrombolysis in the same model, these were 1.6 grams (1.3-1.9) and 4 cm (2.5-4.0), respectively. [Ebben JVS 2014] Four out of five experiments were completed and treated with microbubble infusion through the ultrasound catheter. Unfortunately, one of five pigs died before the start of the experiment due to cardiac and respiratory failure, probably caused by hypersensitivity to the anaesthesia. Since 1 dropout was accounted for in our AEC protocol the experiment was not repeated. In two experiments arrhythmias occurred despite a preventive protocol as previously described [Lab Animal]. One of these experiments was therefore prematurely terminated 15 minutes before the planned end of the experiment.

Discussion

The present manuscript showed that in an in-vitro model the mode of infusion (simulated intra-arterial infusion through the catheter vs. simulated intravenous infusion through the tube lumen of the vascular phantom, see Figure 2) did not influence microbubble survivability or median microbubble size. This suggests that

the impact of the ultrasound of this catheter on microbubble survivability allows for either intravenous- or intra-arterial infusion of microbubbles to accelerate thrombolytic treatment. In addition, the size of the drug delivery lumen and the side holes in the catheter are adequate for intra-arterial infusion of Sonovue microbubbles at a rate of 0.3 mL/minute.

Regarding ultrasound settings, microbubble survivability was significantly different between US settings groups (P<.001) with relative decreases in concentration of 13-41% and 31-35% when the ultrasound setting is ON in the DDC- and tube lumen infusion groups, respectively.

For DDC infusion with ultrasound OFF, a median 94% (70-99, 50um aperture 1000x dilution) and 85% (69-99, 30um aperture, 100x dilution) of the microbubble concentration was measured post infusion. This suggests that microbubbles are able to pass through the ultrasound catheter side holes. For tube lumen infusion with ultrasound OFF, a median 81% (72-91, 50um aperture 1000x dilution) and 74% (55-93, 30um aperture, 100x dilution) of the microbubble concentration was measured post infusion. This loss in concentration could be attributed to mechanical loss, residual concentration in the in-vitro model or measurement error. Nevertheless, the concentration is significantly higher than the concentration of microbubbles which survived in the ultrasound ON group.

If the ultrasound was continuously ON, medians of 58% (37-81, 50um aperture 1000x dilution) and 62% (49-70, 30um aperture, 100x dilution) of microbubble concentration were measured post infusion in the DDC group and medians of 46% (35-60, 50um aperture 1000x dilution) and 42% (28-47, 30um aperture, 100x dilution) in the tube lumen groups. These are significantly lower concentrations compared to the OFF setting (P<.001). This suggests that the effect of continuous ultrasound application results in a median decrease in microbubble concentration after infusion of 41% and 35% (50um aperture, 1000x dilution) and 23% and 32% (30um aperture, 100x dilution) as compared to an OFF setting in the DDC and tube lumen groups, respectively. Additionally, this drop in microbubble concentration with the application of US could indicate potential for a therapeutic effect if the microbubbles were destroyed at the site of interest, i.e. after infusion into a thrombus near the surface of the catheter.

Additionally, the median size of microbubbles changed significantly in the experiments post- vs. pre-infusion with the ultrasound ON setting measured with the 50um aperture (DDC -7.9%, tube lumen -12.7%) and with the 30 um aperture (DDC -22.5%, tube lumen -19.7%). This could imply that ultrasound has more impact on larger microbubbles than smaller ones, with larger microbubbles collapsing and smaller more stable microbubbles forming simultaneously, causing a shift in the size

distribution. Some of the smaller microbubbles sizes measured post infusion may have been debris from the destruction of larger microbubbles rather than actual small microbubbles since the MultiSizer 3 Coulter counter does not distinguish between small bubbles and debris. Nevertheless, the decrease in median size is small and it may not have any clinical significance.

In the present manuscript, we have focused on the feasibility of the combination of microbubbles and an ultrasound catheter for accelerated thrombolysis of peripheral arterial occlusions. As described above, we demonstrated the efficacy of the combined US and microbubbles thrombolytic process in our porcine model. Since we had already performed functional experiments in our porcine model of peripheral arterial occlusion with conventional low-dose CDT we chose to not include the same control group for comparison in the US and microbubble study for ethical reasons. We will, however, compare the results in this discussion section.

The median thrombus weight post-mortem in the present *in vivo* study was 1.02 grams (0.96-1.43); whereas the median thrombus weight in the conventional low-dose CDT group (n=4) in the previous study was 1.59 grams (1.27-1.90). The median thrombus weight in the experimental group in the previous study (US + intravenous microbubbles, n=6) was significantly lower than its controls at a median 1.12 grams (0.83-1.25, P=.01). Regarding remaining thrombus length after 3 hours of treatment, the median length was 2.3 cm (1.5-4.0) in the present study vs. 4.0 cm (2.5-4.0) in the conventional low-dose CDT group of the previous study and 2.5 cm (1.5-4.0) in the experimental group. Although the weights and remaining thrombus lengths of the present and previous experiments cannot be compared directly, these results suggest that treatment with microbubble accelerated low-dose thrombolysis using an ultrasound catheter could potentially accelerate thrombolytic treatment of peripheral arterial occlusions.

The present manuscript is the first study to report experiments utilizing the combination of a clinically used ultrasound catheter and microbubbles approved for administration in humans. It shows both in-vitro data on microbubble survivability, as well as data on the lytic efficacy of this concept in an in-vivo model of peripheral arterial occlusion.

Recently, Gao et al. reported that combining intra-clot microbubble mediated ultrasound cavitation with catheter-directed techniques can significantly improve the efficacy of thrombolysis, both in-vitro and in a rabbit inferior vena cava thrombosis model [ref Gao]. Treatment duration resembled a bolus technique of microbubbles for 10 minutes and thrombolysis up to 60 minutes. Nevertheless, significant lytic effects were observed compared to the controls in this model. On the other hand, quantitative results of the effects of the ultrasound catheter

on microbubble survivability were not reported in the latter study, nor were the effects of clinically available ultrasound catheters on microbubbles approved for clinical use evaluated to this end. In our porcine model of peripheral arterial occlusion, microbubble treatment was performed for 60 minutes and thrombolytic treatment continued for a total of 3 hours.

In the present in-vitro study we used the EKOS ultrasound catheter system that is approved for clinical use and has been shown to accelerate thrombolysis of peripheral arterial occlusions compared to a conventional thrombolysis catheter [DUET 2015]. However, no data is available yet about the effects of this catheter on microbubble survivability when used for microbubble accelerated thrombolysis. The in-vitro data of this study shows that the combination of this catheter and microbubbles is feasible.

The lytic efficacy in our porcine model was evaluated after 3 hours of treatment. This is shorter than the clinical situation of patients with peripheral arterial occlusions with average treatment durations of 2 or 3 days. Significantly longer treatment durations are not practical for ethical and logistic reasons in a porcine model [ref Creager NEJM]. Despite this shorter treatment duration, its effects are significant: median thrombus weights and median lengths of the thrombi post-mortem were significantly lower and shorter compared to the results of conventional catheter-directed thrombolysis in the same model in previous experiments [Ebben JVS 2014].

Limitations of this study

Since this study represents in-vitro and in-vivo models to simulate a clinical technique it is limited by several factors: in the in-vitro setting the tube flow rate (1.0 mL/min) was low compared to arterial flow in order to measure reliable and reproducible concentrations with the coulter counter. However, the lower flow rate resembles a low flow state in a thrombosed artery of a patient. In addition, we didn't account for the influence of blood on the microbubble survivability when they exit the catheter side holes in the in-vitro model. Moreover, this manuscript focuses on feasibility and used small group sizes, which renders preliminary data that doesn't show normality and has variability. Our study warrants future studies to validate these results and to focus on more mechanistic detail. However, our study indicates that microbubble infusion combined with an ultrasound catheter shows significant decrease in microbubble survivability. However, sufficient microbubbles survived to support the feasibility of using this combination to accelerate thrombolysis of peripheral arterial occlusions.

The in-vivo model also has several limitations as previously reported including hyper acute thrombosis, which might influence thrombus composition, and a

shorter treatment time compared to the clinical situation [JVS 2014]. Despite these limitations we believe the set aims of this study were reached and we are able to continue on to the next step of evaluating microbubble accelerated low-dose thrombolysis of peripheral arterial occlusions using an ultrasound catheter.

In conclusion, it is feasible to combine microbubbles with an ultrasound catheter in-vitro and the combination can accelerate thrombolytic treatment of peripheral arterial occlusions in an in-vivo model. However, our study indicates that microbubble infusion combined with an ultrasound catheter shows significant decrease in microbubble survivability. Therefore, future studies are warranted to investigate how to combine the microbubbles with the ultrasound catheter before clinical application.

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Part III

First clinical trial and future perspectives

Chapter 8: Microbubbles and UltraSound-accelerated Thrombolysis (MUST) for peripheral arterial occlusions: protocol for a phase II single-arm trial.

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Abstract

Introduction

Acute peripheral arterial occlusions can be treated with intra-arterial catheter-directed thrombolysis as an alternative to surgical thromboembolectomy. Although less invasive, this treatment is time-consuming and carries a significant risk of hemorrhagic complications. Contrast-enhanced ultrasound using microbubbles could accelerate dissolution of thrombi by thrombolytic medications due to mechanical effects caused by oscillation; this could allow for lower dosages of thrombolytics and faster thrombolysis, thereby reducing the risk of hemorrhagic complications. In this study, the safety and practical applicability of this treatment will be investigated.

Methods and analysis

A single-arm phase-II trial will be performed in 20 patients with acute peripheral arterial occlusions eligible for thrombolytic treatment. Low-dose catheter-directed thrombolysis with urokinase will be used. The investigated treatment will be performed during the first hour of thrombolysis,consisting of intravenous infusion of 4 Luminity vials (1.5 mL each, diluted with saline 0.9% to 40 mL total) of microbubbles with the use of local ultrasound at the site of occlusion. Primary endpoints are the incidence of complications and technical feasibility. Secondary endpoints are angiographic and clinical success, duration of thrombolytic infusion, treatment-related mortality, amputations, additional interventions, and quality of life.

Ethics and disemmination

Ethical approval for this study was obtained in 2015 from the Medical Ethics Committee (METC) of the VU University Medical Center, Amsterdam, the Netherlands. A statement of consent for this study was given by the Dutch national competent authority. Data will be presented at national and international conferences and published in a peer-reviewed journal.

REGISTRATION Dutch National Trial Registry: NTR4731; European Clinical Trials Database (EudraCT) of the European Medicines Agency: 2014-003469-10.

Strenghts and limitations

- This will be a first in man study to examine the safety and technical feasibility of therapeutic microbubbles, combined with the application of ultrasound and catheter-directed thrombolysis in peripheral arterial occlusions.
- This is a ‘single arm’ trial. The data will be used to inform a future large multicentre randomised controlled trial comparing conventional catheter-

directed thrombolysis with microbubble and ultrasound accelerated thrombolysis

- The present study is a non-randomized Phase II-trial, therefore the results cannot confirm benefit of sonothrombolysis for peripheral arterial occlusions, only safety and feasibility is analyzed.
- The present study does not compare other thrombolysis techniques or protocols.

Introduction

Acute limb ischemia can be caused by a thrombus occluding an artery in an arm or leg. This is an emergency situation that can result in amputation or death if not treated successfully.[1] Intra-arterial infusion of thrombolytic agents, i.e. catheter-directed thrombolysis, can restore blood flow by dissolving the clot, as a less invasive alternative to surgical thromboembolectomy.[2] In comparison with the lysis of small arterial occlusions in patients with myocardial infarction, larger peripheral arterial occlusions require higher doses of lytic agents and infusion over a longer period of time. Inevitably, such treatment is accompanied by a risk of major hemorrhagic complications, such as hemorrhagic stroke, in up to 8% of patients.[3] Furthermore, this technique is time-consuming (several days of bed rest is usually required) and repeated angiography for treatment monitoring is needed, putting patients at risk for contrast-induced nephropathy. As a result, this leads to high morbidity rates and a significant patient burden. Methods to improve this therapy are therefore highly sought after.

A potential accelerator of thrombolytic therapy is contrast-enhanced ultrasound. Contrast agents, consisting of 5-10 µm gas-filled particles (microbubbles), have initially been used as diagnostic ultrasound contrast-enhancers. A new field of research investigates these agents for potential therapeutic purposes such as targeted drug delivery and thrombolysis.[4] The proposed mechanism of action in thrombolysis is that microbubbles can oscillate under the influence of ultrasound. At high intensities, this oscillation can lead to microbubble collapse and the production of mechanical forces on the clot surface, making the thrombus more susceptible to thrombolytics, thus accelerating thrombolysis.[5]

In early stages of clinical research, this technique has been shown to be efficient as treatment for acute cerebral stroke and acute myocardial.[6,7] Although the safety of their clinical administration in treating smaller arteries in the heart has been a topic of discussion in the past, post-marketing data for diagnostic indications showed continued safety after extensive research in more recent years.[8,9,10] For therapeutic thrombolytic purposes, this technique has been shown to be effective and safe in a porcine model of large peripheral arterial occlusions.[11] In this study, we will investigate the therapeutic application of microbubbles with ultrasound in combination with catheter-directed thrombolysis for patients with peripheral arterial occlusions. An illustrative video regarding our research project is available as supplementary video.

Methods and analysis

Study objectives

To investigate the safety and practical applicability of the therapeutic application of microbubbles and ultrasound in combination with catheter-directed thrombolysis for patients with peripheral arterial occlusions.

Design

The Microbubbles and UltraSound accelerated Thrombolysis (MUST) trial is a single-arm phase-II trial.

Primary study parameters

Main endpoints will be the safety and technical feasibility of the experimental treatment. Safety will be determined by treatment-related mortality, the occurrence of Adverse Events (AEs), Serious Adverse Events (SAEs) and Suspected Unexpected Serious Adverse Reactions (SUSARs). AEs will be defined as any undesirable experience occurring to a subject during the experimental treatment period, whether or not considered to be related to the investigational drug or intervention. SAEs will be defined as any untoward medical occurrence or effect that at any dose results in death; is life threatening (at the time of the event); requires hospitalization or prolongation of existing in-patients' hospitalization; results in persistent or significant disability or incapacity; is a new event of the trial likely to affect the safety of the subjects, such as an unexpected outcome of an adverse reaction. SUSARs, which are related to the microbubble infusion and ultrasound application, are the formation of micro embolisms resulting in occlusion of the microcirculation, hemorrhages, hypotension, heart rhythm disorders and anaphylaxis. See the paragraph *adverse events* for detailed handling procedures of AEs, SAEs and SUSARs. Hemorrhagic complications related to thrombolytic therapy will be reported according the Standardized Bleeding Definitions for Cardiovascular Clinical Trials proposed by Mehran et al.[12]

Technical feasibility will be defined as accomplishment of the experimental protocol during the first hour of thrombolysis.

Secondary study parameters

Angiographic success will be defined as dissolution of >95% of the thrombus with outflow to at least 1 crural artery. Clinical change/success will be reported according to Rutherford's recommended scale for gauging changes in clinical status (Table I). Amputations will be defined as either major (above or below knee amputation) or minor (metatarsal or toe amputation). Additional interventions will be categorized as either surgical (for example thromboembolectomy, bypass graft surgery) or percutaneous (Percutaneous Transluminal Angioplasty, stent

placement) and as either required for restoration of patency or necessary for correction of underlying lesions. We will also determine microcirculation of the limb (by Laser Doppler measurements, Perimed Instruments, Järfälla, Sweden), 30-day mortality, conversion to surgery, serum fibrinogen concentrations measured during thrombolytic treatment on a daily basis, pain by Visual Analogue Scale (VAS) and quality of life by SF-36 questionnaires. The duration of thrombolysis will be defined by the time-span between initiation and completion angiography.

Table I. Rutherford's recommended scale for gauging changes in clinical status

+3	Markedly improved: No ischemic symptoms, and any foot lesions completely healed; ABI essentially "normalized" (increased to more than 0.90)
+2	Moderately improved: No open foot lesions; still symptomatic but only with exercise and improved by at least one clinical chronic ischemia category; ABI not normalized but increased by more than 0.10
+1	Minimally improved: Greater than 0.10 increase in ABI* but no categorical improvement or vice versa (i.e., upward categorical shift without an increase in ABI of more than 0.10)
0	No change: No categorical shift and less than 0.10 change in ABI
−1	Mildly worse: No categorical shift but ABI decreased more than 0.10, or downward categorical shift with ABI decrease less than 0.10
−2	Moderately worse: One category worse or unexpected minor amputation
−3	Markedly worse: More than one category worse or unexpected major amputations

*In cases where the ABI cannot be accurately measured, an index based on the toe pressure, or any measurable pressure distal to the site of revascularization, may be substituted.

Adapted from: Rutherford RB, Baker JD, Ernst C, Johnston KW, Porter JM, Ahn S, et al. Recommended standards for reports dealing with lower extremity ischemia: revised version. J Vasc Surg. 1997;26(3):517–38.
Abbreviations: ABI=Ankle Brachial Index

Patients and eligibility criteria

The present feasibility and safety study is a non-randomized Phase II trial, to be conducted in our university hospital in Amsterdam, the Netherlands. Usually in a phase-II trial, 10-20 patients are investigated to confirm an occurrence of toxic effects or serious adverse events <20%. We chose a sample size of 20 to assess the safety of the investigational treatment. Eligibility criteria are listed in Table II. Inclusion of 20 eligible patient is expected within 1.5 years. Written informed

consent will be acquired by a member of the Research Team after information about the study has been provided by the treating doctor.

Table II. Eligibility criteria

Inclusion criteria	• Men and women older than 18 and younger than 85 years old • Patients with a maximum of 2 weeks of symptoms for lower limb ischemia due to thrombosed/occluded iliofemoral, femoropopliteal or femorocrural native arteries or iiliofemoral, femoropopliteal or femorocrural venous or prosthetic bypass grafts • Patients appropriate for thrombolysis i.e. with acute lower limb ischemia class I and IIa according to the Rutherford classification • Patients who understand the nature of the procedure and provide written informed consent before enrollment in the study
Exclusion criteria	• Patients with clinical complaints of acute lower limb ischemia due to thrombosis of iliofemoral, femoropopliteal or femorocrural native arteries, or iliofemoral, femoropopliteal or femorocrural venous or prosthetic bypass grafts for more than 2 weeks • Patients with thrombosed popliteal aneurysms • Patients with absolute contraindications for administration of antiplatelet therapy, anticoagulants or thrombolytics • History of recent (less than 6 weeks) ischemic stroke, cerebral hemorrhagic or myocardial infarction • Patients with recent (less than 6 weeks) surgery • Severe hypertension (diastolic blood pressure greater than 110 mm Hg, systolic blood pressure higher than 200 mm Hg) • Current malignancy or severe co-morbid condition with a life expectancy of less than 6 months • Patients with uncorrected bleeding disorders (gastrointestinal ulcer, menorrhagia, liver failure) • Women with childbearing potential not taking adequate contraceptives or currently breastfeeding • Pregnancy • Patients who are currently participating in another investigational drug or device study • Patients younger than 18 years or older than 85 years • Patients with contraindications for Luminity microbubbles i.e.: • Hypersensitivity to perflutren or to any of the components of Luminity • Recent acute coronary syndrome or clinically unstable ischemic cardiacdisease, including: evolving or ongoing myocardial infarction, unstable angina at rest within the last 7 days, significant worsening of cardiac symptoms within the last 7 days, recent coronary artery intervention or other factors suggesting clinical instability (for example, recent deterioration of ECG, laboratory or clinical findings), acute cardiac failure, Class III/IV cardiac failure, or severe rhythm disorders • Patients known to have right-to-left cardiac shunts, severe pulmonary hypertension (pulmonary artery pressure >90 mmHg), uncontrolled systemic hypertension and in patients with GOLD Stage IV COPD, diffuse interstitial fibrosis or adult respiratory distress syndrome • Patients with cardiovascular instability where dobutamine is contraindicated

Data handling

We will keep an electronic log of patients who fulfill the eligibility criteria, patients who are invited to participate in the study, patients recruited and patients who withdraw from the study. Reasons for non-recruitment will also be recorded. We will attempt to collect reasons for non-participation from patients who decline to take part. During the course of the study, we will document reasons for withdrawal from the study and loss to follow-up. Data will be stored electronically in Case Report Forms software with audit trail functionality and will be audited by the institutional Clinical Research Bureau (CRB). Only anonymized information will be stored and participants will only be identifiable by their unique study number, which will be kept in a separate file. Data will be securely stored on these servers for 15 years according to national guidelines. The principal investigator will have access to the final trial dataset. No independent Data Management Committee was instated according to local ethics committee guidelines since the present study was not classified as a high-risk clinical study. This classification was approved by the local ethics committee based on the risk assessment form of the Netherlands Federation of University Medical Centres.

Study procedures

Intervention

A flow chart of the patient work-up after presentation in our hospital is presented in Figure 1. Low-dose thrombolytic treatment with urokinase will be initiated following our standard institutional protocol: a catheter is placed intra-arterially in the affected artery and a bolus injection of 500,000 International Units (IU) of urokinase (Medacinase Urokinase, Medac GmbH, Hamburg, Germany) will be followed by the continuous infusion of 50,000 units of UK per hour and 9,600 IU of heparin per 24 hours. The experimental treatment consists of (in addition to the standard thrombolytic therapy) the use of local 1.8 Mhz transdermal ultrasound (Philips iE33 Ultrasound Machine, Eindhoven, the Netherlands), and the intravenous infusion of 4 Luminity vials (total 6 mL, diluted with saline 0.9% to 40 mL total, Lantheus MI UK Limited, Newbury, Berkshire, United Kingdom) during the first hour of thrombolysis with urokinase. An ACIST VueJect (Bracco Imaging Europe B.V., the Netherlands) infusion pump will be used to infuse the 4 vials continuously. Ultrasound will be intermittently activated (3 seconds manual flash to burst microbubbles with Mechanical Index (MI) 1.08 (pulse duration 20 microseconds, frequency 1.8 Mhz, framerate 39 Hz), 7 seconds of visualization of inflow of the microbubbles at MI±0.11, at the site of occlusion during the first hour of thrombolysis. Criteria for discontinuation of the experimental treatment during the first hour will be the occurrence of any adverse events potentially related to the experimental treatment such as bleeding and allergic reactions.

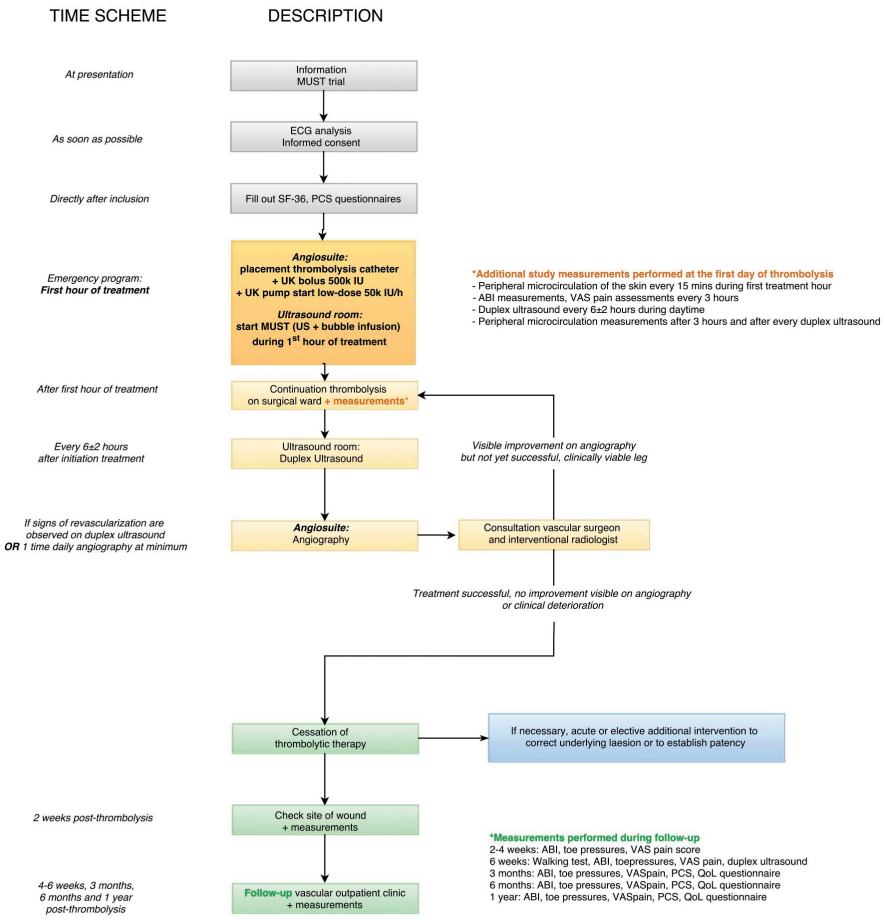

Figure 1. Flow chart of patient work-up after presentation

Assessments

Diagnostic measurements

Additional diagnostic measurements during admission including ECG, duplex ultrasound, angiography and microcirculation measurements (by Laser Doppler flowmetry) will be performed as depicted in Figure 1.

A duplex ultrasound will be performed every 6±2 hours to monitor for signs of revascularization. When resumption of flow is visualized by duplex ultrasound, angiography will be performed to confirm flow. Angiography will be performed at

least once daily as standard procedure. Outside of routine hospital working hours, angiography will only be performed in emergencies as per standard care.

A standardized pain score (Visual Analogue Scale, 1-10), and Pain Catastrophizing Scale will be recorded every 3 hours by a nurse practitioner, research fellow or surgical resident to assess pain.

Fibrinogen monitoring

Following our standard institutional thrombolysis protocol, fibrinogen concentration will be checked during thrombolysis with the following criteria for treatment modification: If <1.0 g/L, the urokinase infusion rate will be lowered to 25,000 IU/h; if <0.5 g/L, thrombolysis must be aborted temporarily and replaced by normal saline infusion. Three hours following treatment modifications, fibrinogen concentration will be reevaluated and when >1.0 g/L thrombolysis will be restarted at an initial low dose urokinase of 50,000 IU/h.

Post-procedural anticoagulation

After successful thrombolysis, the patient will be heparinized with low-molecular weight heparin (fraxiparine) dosed based on body weight: <50 kg: 2 times a day 3,800 IU (= 0.4 mL), 50-80 kg: 2 times a day 5,700 IU (= 0.6 mL), >80 kg: 2 times a day 7,600 IU (= 0.8 mL).

Concomitant therapy with coumarin derivatives will also be started at that time. Activated partial thromboplastin time (aPTT) will be measured daily during heparin treatment. The target range international normalized ratio (INR) will be 2.5 to 3.5; if this value is reached, heparinization will be stopped and coumarin treatment will be continued.

Follow-up

Outpatient follow-up will take place at specific time points for a total duration of 1 year, measurements performed during follow-up visits are depicted in Figure 1.

Adverse events

Adverse events will be recorded in detail in the electronic patient record. Any serious adverse events that occur after joining the trial will be reported to the accredited Medical Ethics Committee of our institution according to national and institutional guidelines. All adverse events will be followed up until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to a general physician or medical specialist. An interim analysis after 10 patients will be performed and if serious adverse events have occurred, we will discuss the

continuation of the study. The study will be prematurely terminated if 2 or more intracranial bleedings occur or more than 5 allergic reactions.

Statistical analysis

Categorized epidemiologic/descriptive patient variables are summarized with frequencies and will be analyzed with Fischer's exact test or the Pearson Chi-squared test. To avoid possible violations of the assumptions for parametric testing, such as a normal distribution pattern, we will employ non-parametric methods such as a Spearman rank correlation and a Mann-Whitney U test in the case of a skewed distribution or log transformation. For associations of two outcome measurements, we will use a correlation (Spearman rank) or single regression analysis. We will analyze the following outcomes by means of Kaplan-Meier curves: patency rate, amputation-free rate, and intervention-free rate. We will assess heterogeneity in prognostic factors as a secondary analysis by means of Chi-squared tests. All tests will be performed two-sided, and a $p<0.05$ will be considered to be statistically significant.

Ethics and disemmination

The study will be conducted according to the principles of the Declaration of Helsinki (Brazil, October 2013), and in accordance with the Medical Research Involving Human Subjects Act (WMO). An Investigator Site File will be produced in advance of the study conforming to institutional guidelines. Furthermore we will create Case Report Forms by using GCP and 21 CFR Part 11 compliant software, to handle patient data.

The study has been registered in the Dutch Trial Register, at the Dutch National Central Committee on Research Involving Human Subjects (CCMO) and in the European Clinical Trials Database (EudraCT) of the European Medicines Agency. Any protocol amendments during the study will be communicated and changed accordingly in the relevant registries after approval of the institutional Medical Ethics Committee. The results of this study will be submitted for publication in a peer-reviewed journal, regardless of the outcome of this study, according to the CCMO statement on publication policy. Data will also be presented at international conferences.

Discussion

The Microbubbles and UltraSound-accelerated Thrombolysis (MUST) trial is a phase-II single-arm clinical trial. In this study the safety and feasibility of an experimental ultrasound technique will be investigated for the first time in patients with large peripheral arterial occlusions.

We believe that this procedure is safe and can accelerate thrombolysis, thereby allowing for reduction of thrombolytic dosage, which in turn reduces the risk of major hemorrhagic complications.

An experimental bolus therapy with microbubbles and ultrasound could accelerate thrombolysis because at high ultrasound intensities microbubbles can collapse, resulting in mechanical forces on the clot surface. The formation of small channels in the thrombus lead to exposure of a larger total surface susceptible to thrombolytics.[5]

In regard to the therapeutic application of contrast agents, several studies have been performed in patients with ischemic stroke and myocardial infarction. A systematic review of sonothrombolysis shows that this treatment option improves clinical and long-term outcomes, while potentially reducing bleeding risk, in patients with ischemic stroke.[13] Nevertheless, dose escalation studies show that the safety (in terms of bleeding and micro emboli) needs to be further investigated before enrolling patients in phase-III trials.[14] Few and heterogeneous studies examined the therapeutic application of sonothrombolysis in patients with myocardial infarction. Although pilot studies affirm safety and feasibility, the application of therapeutic ultrasound with longer pulse durations (20 microseconds vs. 5 microseconds) was reported to result in unexpected coronary vasoconstriction in a recent clinical trial.[15]

Potential reported mechanisms for this effect are the summative effect of myocardial ischemia, reperfusion damage and long-pulse-duration sonoporation on endothelial damage, all leading to calcium overload.

However, patients with peripheral arterial occlusions are mostly chronic vascular patients who often have received previous treatments in the respective artery, for example thrombolytic therapy, percutaneous transluminal angioplasty, thromboembolectomy or bypass surgery. The mechanical manipulation of the vascular wall during all these treatments is extensive. Furthermore, during standard thrombolytic treatment, arteries are manipulated and perforated on purpose to insert guide wires and catheters. Hence, vascular spasms during these peripheral treatments are normal and non-threatening to the patient, in contrast to spasms in small coronary arteries.

The administration of ultrasound contrast agents has been accompanied by important discussions regarding safety concerns in the past[8,16]. As a response to the occurrence of SAEs, the US Food and Drug Administration (FDA) issued a labeling change and warnings for contrast agents in 2007. Consequently, new studies on the risks of contrast agents were performed and these established their safety.[17]

In regard to Luminity contrast agent dose regimens, the recommended dose for diagnostic indications is 1.3 mL dispersion added to 50 mL of sodium chloride 9 mg/mL (0.9%) or glucose 50 mg/mL (5%) solution injected over a short time-period.[18] For therapeutic purposes, in large peripheral arteries there are no dose studies available. However, in our university hospital the Sonolysis study has been performed by our Cardiology department to treat acute ST elevation myocardial infarction patients with Luminity microbubbles and high mechanical ultrasound.[19] The dose used was one flacon Luminity of 1.5 mL which contains 225 microliter perflutren diluted with 48.5 mL of saline 0.9% to create a 50 mL suspension. Patients were treated with 15 minutes with an infusion rate of 200 mL/h. No serious adverse events occurred. In the present study to establish a therapeutic effect in large arterial occlusion we will also infuse 1 vial per 15 minutes but we will treat patients for 60 minutes. We will use 4 flacons of 1.5 mL Luminity containing 900 microliter perflutren diluted with saline 0.9% to 40 mL total volume to be infused during 1 hour. The clinical consequences of overdose with Luminity are not known. Single doses of up to 100 microlitres dispersion/kg and multiple doses up to 150 microlitres dispersion/kg were tolerated well in Phase I clinical trials.[20] This equals to the infusion of 12 mL (8 flacons) of Luminity dispersion. We will administer a total of 6 mL (4 flacons) of Luminity dispersion. Furthermore, we will not administer them as single bolus doses but as low-speed continuous infusion. During the experimental protocol with microbubble infusion, patients will be continuously monitored.

As with all contrast agents, the risk of anaphylactic reactions to contrast remains. Therefore, administration of contrast agents in a center with full resuscitation possibilities is mandatory. Furthermore, during the first hours of administration, monitoring of vital parameters of patients is important.

In this study, thrombolysis is performed with the fibrinolytic urokinase, which is the most used fibrinolytic agent for the treatment of peripheral arterial occlusions worldwide and is standard care in the Netherlands. Some countries use tissue plasminogen activator for this indication. A Cochrane review on the topic states that there is no evidence that (r)t-PA is more effective than urokinase for patients with peripheral arterial occlusion.[21]

If the application of microbubbles and ultrasound concomitant to catheter-directed thrombolysis is shown to be safe and technically feasible based on this phase-II trial, we anticipate a funding application for a larger randomized controlled trial with a comparative group to assess and compare efficacy of this treatment.

Although the efficacy of the currently described protocol cannot be adequately compared within this study design, we will discuss the outcomes relative to a historic

control group that had previously received our standard hospital thrombolysis protocol.[22]

Successful thrombolysis is strongly predictive of amputation-free survival with vascular patency for at least one year.[23] A longer duration of thrombolysis inevitably exposes a patient to a higher thrombolytic dose and higher risk of hemorrhage, in addition to an already increased patient burden because of prolonged bed rest. Therefore ultimately, acceleration of thrombolysis with microbubbles could benefit the patient because of a shorter therapy time, a lower risk of hemorrhagic complications and a decrease in patient burden.

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Author contributions
HE, JN, WW and KK planned and designed the research, HE wrote the manuscript, HE, JN, RL and KK critically revised the manuscript and all authors and MUST collaborators approved the final version of the manuscript.

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Competing interest
None.

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Chapter 9: Microbubbles and UltraSound-accelerated Thrombolysis (MUST) for peripheral arterial occlusions: the outcomes of a phase II single-arm trial

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Abstract

Objectives

Acute peripheral arterial occlusions can be treated by catheter-directed thrombolysis (CDT). However, CDT takes time and is accompanied by major haemorrhagic complications. The addition of contrast-enhanced ultrasound and microbubbles could improve thrombus susceptibility for thrombolytic agents, potentially shorten treatment time with less risk of bleeding complications. This article reports the outcomes of the safety and feasibility of the use of microbubbles contrast-enhanced sonothrombolysis in patients with acute peripheral arterial occlusions.

Methods

In this single-arm phase-II-trial, 20 patients with Rutherford I and IIa acute lower limb ischemia received CDT combined with an intravenous infusion of microbubbles and locally applied ultrasound during the first hour of the standard intra-arterial thrombolytic therapy. Primary endpoint was safety, i.e. occurrence of serious adverse events (haemorrhagic complications and/or amputation) and mortality within 1 year. Secondary endpoints included angiographic and clinical success, thrombolysis duration, additional interventions, conversion and quality of life.

Results

The study included 20 patients (16 men; median age, 68.0 (range, 50.0-83.0) years and 40% native artery and 60% bypass occlusions). In all patients, the use of microbubbles contrast-enhanced sonothrombolysis could be successfully applied. No serious adverse events related to the experimental treatment occurred. Duplex examination showed flow distal from occlusion after 23.1 hours (range 3.1-46.5) with a median thrombolysis time of 47.5 hours (range, 6.0-81.0). The short-term ABI and pain-scores significantly improved, however no changes were observed before and after thrombolysis in the microcirculation. Overall mortality and amputation rates were both 2% within 1-year. The 1-year patency rate was 55%.

Conclusions

The results of the MUST-trial show that treatment of patients with acute peripheral arterial occlusions with microbubbles enhanced sonothrombolysis is feasible and safe to perform in patients. Further research is necessary to investigate the superiority of this new treatment over standard treatment.

Keywords

Peripheral arterial occlusion, catheter directed thrombolysis, microbubbles, sonothrombolysis, ultrasound, phase-II-trial

Introduction

Several studies during the 1990s have revealed that catheter-directed thrombolysis (CDT) and surgical interventions were equivalent treatment options for acute peripheral occlusions, mostly in terms of amputation free survival in up to one year(1). Contrary to surgical intervention, infusion of thrombolytic agents has the ability to treat small occluded side branches and, if needed, simplify any required procedures to correct unmasked lesions(2). While thrombolysis showed to be a good alternative to surgical treatment, this procedure is still accompanied by major haemorrhagic complications in up to 8.4%(3). Paradoxically, limb ischemia may even worsen during thrombolytic treatment due to distal embolization of the clot. Besides the associated complications, this treatment needs monitoring by repeated angiography, which entails the risk of nephrotoxicity. In short, optimization of the current thrombolytic treatment is needed to minimize complication occurrences, limit patient burden and improve outcome.

Microbubbles 1-10 µm in size were initially developed as ultrasound contrast agents to improve conventional ultrasound scanning(4). However, several studies have addressed the potential of microbubbles combined with ultrasound in thrombolysis. The second generation of ultrasound contrast agents consists of a high-molecular-weight gas encapsulated in a lipid shell, used to improve stabilization and thereby making the microbubbles capable of transpulmonary passage into the arterial circulation. During continuous intravenous infusion, microbubbles can be visualized as an echogenic area for several minutes before their shell is metabolized to free fatty acids and the gas is eliminated from the systemic circulation via the lungs(5). The generally accepted idea is that low and intermediate acoustic pressure causes oscillation of the microbubbles which results in collapse under high acoustic pressure. Destruction of these microbubbles can lead to the formation of free radicals, causing erosion and small holes in the surface of the clot which weakens the fibrin network and eventually increases susceptibility of the clot for the fibrinolytic agent(6).

The effectiveness of the therapeutic application of contrast-enhanced sonothrombolysis (CEST) has already been demonstrated in clinical trials for treatment of acute stroke and myocardial infarction(7). Previous studies in a porcine model of large peripheral arterial occlusion, have shown beneficial effects of this technique(8). To date, this therapy has never been tested in the setting of large peripheral occlusions in humans. The objective of this study is to investigate the safety and practical application of intra-arterial infusion of thrombolytic agents combined with microbubbles and ultrasound in peripheral arterial occlusions.

Methods

Study design and patients

The protocol for this trial has been previously described in BMJ open (9). In brief, the Microbubbles and UltraSound-accelerated Thrombolysis (MUST) trial, a single-arm phase II trial, was conducted between July 2017 and November 2019 in the Amsterdam UMC location VUmc the Netherlands. Patients were also referred from Dijklander Hospital, Hoorn in the Netherlands to be included in the MUST-trial in Amsterdam UMC.

A sample size of 20 patients with acute lower limb ischemia Rutherford Classification I or IIa was chosen to assess the safety of the novel treatment. A detailed list of the eligibility criteria is given in Table 1. All of the patients were informed about CEST by the treating physician and written informed consent acquired.

The institutional review board approved the trial protocol and the study was performed in accordance with the Declaration of Helsinki. The trial has been registered in the Dutch Trial Register, at the Dutch National Central Committee on Research Involving Human Subject (registration number: NTR4731) and in the European Clinical Trials Database of the European Medicines Agency (registration number: 2014-003469-10).

Intervention

In advance of the thrombolysis treatment, unfractionated heparin was given systematically to inhibit thrombus propagation and was discontinued when CDT was initiated.

Due to market manufacturing problems of Urokinase in May 2018, half of the group was treated with Urokinase (UK) until October 2018 and thereafter with recombinant tissue plasminogen activator (rtPA). A sheath was inserted in the left or right common femoral artery under ultrasound guidance. A McNamara catheter was placed intra-arterially in the distal segment of the thrombus. After successful positioning of the thrombolysis catheter, the first 10 patients received the standard low dose thrombolysis protocol consisting of a bolus injection of 500 000 International Units (IU) of Urokinase followed by the continuous infusion of 50 000 IU of UK per hour. The last 10 patients received a bolus of 5 mg of Alteplase, followed by the continuous infusion 1 mg rtPA per hour for 24 hours and 0.5 mg rtPA after 24 hours. Additionally, a continuous dose of 4800 IU of heparin per 24 hours was infused through the side-port of the sheath to prevent clot formation at the catheter. In addition to the standard thrombolytic therapy, patients received continuous intravenous infusion of 4 vials of microbubbles (Luminity, Lantheus MI UK, Newburry, Berkshire, UK; total 6 mL, diluted with 0.9% to 40 mL total)

Table 1. The eligibility criteria

Inclusion criteria	• Men and women older than 18 and younger than 85 years • Patients with a maximum of 2 weeks of symptoms for lower limb ischaemia due to thrombosed/occluded iliofemoral, femoropopliteal or femorocrural native arteries or iliofemoral, femoropopliteal or femorocrural venous or prosthetic bypass grafts • Patients appropriate for thrombolysis, that is, with acute lower limb ischaemia class I and IIa according to the Rutherford classification • Patients who understand the nature of the procedure and provide written informed consent before enrolment in the study
Exclusion criteria	• Patients with clinical complaints of acute lower limb ischaemia due to thrombosis of iliofemoral, femoropopliteal or femorocrural native arteries, or iliofemoral, femoropopliteal or femorocrural venous or prosthetic bypass grafts for >2 weeks • Patients with thrombosed popliteal aneurysms • Patients with absolute contraindications for administration of antiplatelet therapy, anticoagulants or thrombolytics • History of recent (<6weeks) ischaemic stroke, cerebral haemorrhagic or myocardial infarction • Patients with recent (<6weeks) surgery • Severe hypertension (diastolic blood pressure >110 mmHg and/or systolic blood pressure >200 mmHg) • Current malignancy or severe comorbid condition with a life expectancy of <6 months • Patients with uncorrected bleeding disorders (gastrointestinal ulcer, menorrhagia, liver failure) • Women with childbearing potential not taking adequate contraceptives or currently breast feeding • Pregnancy • Patients who are currently participating in another investigational drug or device study • Patients younger than 18 years or older than 85 years • Patients with contraindications for Luminity microbubbles, that is: • Hypersensitivity to perflutren or to any of the components of Luminity • Recent acute coronary syndrome or clinically unstable ischaemic cardiac disease, including evolving or ongoing myocardial infarction, unstable angina at rest within the last seven days, significant worsening of cardiac symptoms within the last seven days, recent coronary artery intervention or other factors suggesting clinical instability (e.g., recent deterioration of ECG, laboratory or clinical findings), acute cardiac failure, class III/IV cardiac failure or severe rhythm disorders • Patients known to have right-to-left cardiac shunts, severe pulmonary hypertension (pulmonary artery pressure >90 mmHg), uncontrolled systemic hypertension and in patients with Global Initiative for Obstructive Lung Disease (GOLD) stage IV chronic obstructive pulmonary disease, diffuse interstitial fibrosis or adult respiratory distress syndrome • Patients with cardiovascular instability where dobutamine is contraindicated

during the first hour of thrombolysis. An ACIST VueJect (Bracco Imaging Europe B.V., Amsterdam, the Netherlands) infusion pump was used to infuse the four vials continuously. Transdermal ultrasound (Philips iE33 Ultrasound Machine, Eindhoven, the Netherlands) was directed at the site of occlusion during the first hour of thrombolysis and intermittently activated (3s manual flash to burst microbubbles with Mechanical Index 1.08 (pulse duration 20 μs, frequency 1.8 MHz, frame rate 39 Hz), 7s of visualization of inflow of microbubbles at MI ±0.11).

Patient were routinely treated on a standard surgical ward where fibrinogen levels were monitored following local protocols at that time. A control angiography was performed every 24 ± 2 hours to evaluate progression of thrombolysis and to determine continuation of therapy with or without additional interventions.

Endpoints and definitions

The primary endpoints were the safety and technical feasibility of the contrast enhanced sonothrombolysis. Safety was determined by mortality, the occurrence of adverse events (AEs), serious adverse events (SAEs) and suspected unexpected serious adverse reactions (SUSARs). The study would have been terminated earlier if two or more intracranial bleedings or more than five allergic reactions occurred. Technical feasibility was defined as accomplishment of the experimental protocol after the first hour of thrombolysis.

Secondary endpoints included clinical change/success according to Rutherford's recommended scale for gauging changes in clinical status (Table 2) (10); additional measurements (transdermal microcirculation, serum fibrinogen concentrations, pain-scores); duration of thrombolytic therapy needed for complete lysis and additional interventions. Complete lysis was defined as complete resolution of the thrombosed native artery or bypass graft with restoration of inflow and outflow to at least one crural artery.

Measurements

Transdermal microcirculation of the limb was measured every 15 minutes during the first hour of treatment and thereafter every 3 hours up until regain of flow was visualized, from which point the measurements were performed every day. Every 6 hours a duplex ultrasound was performed to monitor for signs of revascularization. According to standard procedure an angiography was performed at least once a day.

Table 2. Rutherford's recommended scale for gauging changes in clinical status.

+3	Markedly improved: no ischaemic symptoms and any foot lesions completely healed; ABI essentially 'normalised' (increased to >0.90)
+2	Moderately improved: no open foot lesions; still symptomatic but only with exercise and improved by at least one clinical chronic ischaemia category; ABI not normalised but increased by >0.10
+1	Minimally improved: >0.10 increase in ABI but no categorical improvement or vice versa (i.e., upward categorical shift without an increase in ABI of >0.10)
0	No change: no categorical shift and <0.10 change in ABI
-1	Mildly worse: no categorical shift but ABI decreased >0.10 or downward categorical shift with ABI decrease <0.10
-2	Moderately worse: one category worse or unexpected minor amputation
-3	Markedly worse: more than one category worse or unexpected major amputations

To document objective change, Ankle-branchial indices (ABIs) of both legs were measured preprocedural, postprocedural and every 3 hours during the treatment up until reconstitution of flow was visualized, from which point the measurements were performed every day to document objective change. Furthermore, a standardized pain score (VAS, 1-10) was recorded every 3 hours by a research fellow to assess pain.

Follow-up

The follow-up consists of a visit to the outpatient clinic, with clinical examination including ankle-branchial indices (ABI), pain-free walking distance, duplex ultrasound of the treated limb and questionnaires regarding the quality of life (Fig. 1). During follow-up, any cases of recurrence of thrombosis, reinterventions or amputation on the same limb and mortality of the patients were recorded.

Statistical analysis

The group differences were determined by an independent t-test or a Mann-Whitney U test in case of a skewed distribution. A Wilcoxon signed rank test was used to compare the improvement in continuous variables before and after intervention in both groups. Multiple related samples were tested with Friedman's ANOVA and using the Wilcoxon signed rank test for post-hoc analysis. The patency rate was analysed by means of the Kaplan-Meier method. A P-value of <.05 (two-sided) was considered statistically significant. Statistical analyses were performed with SPSS (IBM Statistics v20, Armonk, NY).

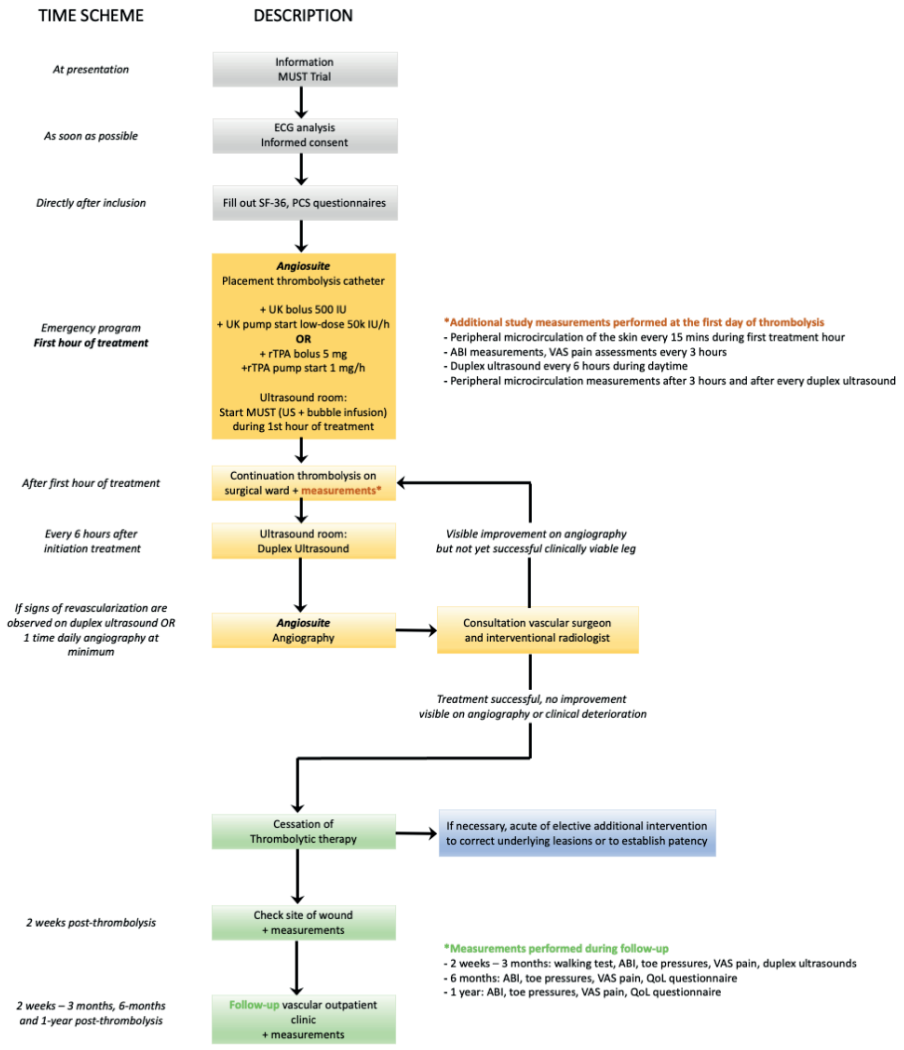

Figure 1. Study flow chart of patient work-up after presentation with acute peripheral arterial occlusion.

ABI: Ankle Branchial Index, IU: International Units, MUST: Microbubbles and UltraSound-accelerated Thrombolysis, QoL: Quality of Life, UK: Urokinase, rtPA: Alteplase, US: ultrasound, VAS: visual analogue scale.

Results

Contrast-enhanced thrombolysis for thromboembolic obstructions in peripheral arteries was performed in 20 patients, of which 10 were treated with UK and 10 with rtPA. Baseline characteristics of the included patients and occlusions characteristics are listed in Table 3.

Primary outcomes

Contrast enhanced thrombolysis was technically feasible in all 20 patients. In terms of the primary safety outcome, no major haemorrhagic complications nor any events of systemic allergic reactions, in-hospital mortality or 30-day mortality related to the microbubbles or ultrasound could be reported.

Secondary outcomes

Secondary outcomes were analysed in 19 patients. One patient was excluded from statistical analysis after receiving microbubbles therapy, as the pre-treatment plan was to use contrast enhanced sonothrombolysis for rapid restoration of blood flow as well as to facilitate an easier access for the surgical procedure of underlying vascular lesions. Due to insufficient file documentation, the analysis of the VAS scores, microcirculation and ABI measurements of another patient could not be performed.

Secondary outcomes regarding treatment characteristics and results are summarized in Table 4. Median time until return to flow at duplex ultrasound was established after 23.1 hours (range 3.1-46.5 hours). In nine patients (50%), return to flow at duplex ultrasound was achieved within 24 hours. Median time until revascularisation at angiography was established within 25.5 hours (range 6.0-81.0 hours). Median duration of total thrombolytic therapy was 47.5 hours (range 6.0-81.0 hours).

Angiographic patency was reached in all cases. In 74% of all cases (n=14) additional percutaneous interventions were required to treat underlying stenotic lesions (e.g., Percutaneous Transluminal Angioplasty (PTA) with or without stent placement). In four cases additional surgical interventions were performed, in one case a femoral-popliteal bypass and in three cases thromboembolectomy. All patients were discharged with a patent treated native artery or bypass graft after additional procedures. After thrombolysis, clinical improvement was observed in 85% (n=17) of all cases. One case in the UK group had mildly worsened in clinical status due to a poor distal run-off which resulted in an early reocclusion of the target artery.

Table 3. Baseline and occlusion characteristics.

	Total	UK patients (n=10)	rtPA patients (n=10)	p*
Age, year in mean (standard deviation)	69.2±2.1	70.8±7.9	67.6±10.9	.462
Male sex – no.	16	8	8	1.00
SVS/ISCVS risk-factor score – no.				
Diabetes	3	0	3	.068
Tobacco use	7	4	3	.834
Hypertension	10	2	8	.131
Hyperlipemia	15	6	7	1.00
Cardiac disease	3	1	2	1.00
Carotid-artery disease	0	0	0	1.00
Renal disease	4	0	4	.029
Pulmonary disease	2	1	1	.942
Sum of SVS/ISCVS-risk-factor scores †	5.05±2.57	4.40±2.01	5.70±2.98	.268
Medication at admission				
Anti-platelets	7	3	4	.473
Coumarins	5	3	2	.615
Non-vitamin K oral anticoagulant	5	2	3	.615
Heparins	0	0	0	1.00
Type of occlusion				
Native	8	5	3	.374
Bypass	13	5	7	.661
Venous	1	1	0	.317
Prosthetic	8	3	5	.374
Stented native artery	3	1	2	.542
Number of occlusions				
1 occlusion	16	8	8	1.00
2 occlusions	4	2	2	1.00
Duration of symptoms, days in median (range)	6,0 (0-14)	6,5 (0-14)	4,0 (3-14)	.593
Rutherford classification				
Rutherford class I	3	2	1	.542
Rutherford class IIA	17	8	9	.542
Previous revascularization same limb				
PTA and/or stenting	13	6	7	.897
Thrombolysis	10	5	5	.579
Bypass	6	3	3	1.00
Thrombo-embolectomy	5	4	1	.684

Data are presented as *n* or mean ± standard deviations (SD) or median (interquartile range [IQR]). UK=Urokinase, rtPA=Alteplase, PTA=percutaneous transluminal angioplasty.

* *p* value of difference between thrombolytic agents.

† The Society for Vascular Surgery/International Society for Cardiovascular Surgery (SVS/ISCVS) risk-factor score for each of eight domains ranges from 0 (no risk factors) to 3 (severe risk factors). Total scores can range from 0-24, with higher score indicating more risk factors.

Table 4. Secondary outcomes.

	Total	UK patients (n=10)	rtPA patients (n=10)	p *
Treatment details, in hours (range)				
Time to				
Flow at duplex examination	23.1 (3.1-46.5)	24.0 (5.0-46.5)	8.3 (3.1-29.5)	.310
Revascularisation at angiography	25.5 (6.0-81.0)	51.0 (6.0-81.0)	24.8 (20.3-73.2)	.216
Total thrombolysis duration	47.5 (6.0-81.0)	53.5 (6.0-81.0)	27.7 (20.3-73.2)	.216
Total UK dose administered (IU x 106)	-	4.3 (3.0-9.1)	-	
Total rtPA dose administered (mL)	-	-	38,0 (18,5-54,2)	
Additional procedures				
PTA / stenting	19	7	12	.372
Thrombo-embolectomy	3	2	1	.542
Rutherford gauging scale of clinical change between admission and at discharge				
Markedly improved – n	8	4	4	1.00
Moderately improved – n	6	3	3	1.00
Minimally improved – n	3	2	1	.317
No change – n	0	0	0	1.00
Mildly worse – n	1	1	0	.317
Moderately worse – n	0	0	0	1.00
Markedly worse – n	0	0	0	1.00
Fibrinogen (in g/dL, mean ± SD)	2.37 ±.69	2.38 ±.92	2.37 ±.43	.995
APT (in s, mean ± SD)	46 ±.12.0	46 ±.10.8	46 ±.13.7	.996
PTT (in INR, mean ± SD)	1.23 ±.34	1.21 ±.29	1.24 ±.41	1.00

Data are presented as *n* or mean ± standard deviations (SD) or median (interquartile range [IQR]). UK=Urokinase, rtPA=Alteplase, PTA=percutaneous transluminal angioplasty; APTT=activated partial thromboplastin time; PTT=partial thromboplastin time.

* *p* value of difference between thrombolytic agents.

Although no major bleeding complications occurred in any of the groups, minor bleeding at the sheath insertion site or groin hematoma was noted in three cases (16%). None of these patients required volume substitution or surgical evacuation. All cases with minor bleeding complications showed APTT and fibrinogen values within normal ranges, whereas in one patient the INR was slightly elevated 1.25 g/dL on the day of the bleeding complication.

Ankle branchial index increased significantly after treatment in 89% of all patients (.27 mL/min (range .0 - .81) vs .82 mL/min (range .29 - 1.20), p=.00; Fig. 2). A non-significant increase in microcirculation was observed in 61% of all patients (p=.732). VAS-scores were significantly lower after treatment in the whole group (p=.005).

Outcomes during follow-up

Median duration of follow-up was 12-months (range 8.9 – 12). One patient died at 8-months due to (pre-existent) cardiac failure and a concomitant refusal of further therapy. The median ABI at discharge was .82 mL/min (range .29-1.20), increased to .94 mL/min (range .29-1.23) 6-weeks after the intervention and decreased to .84 mL/min (range .46-1.16) at 12-months follow-up (Fig. 2).

During the 12-months follow-up, 11 patients experienced re-occlusions and 2 stenosis. The primary patency rates at 3-, 6- and 12-months were 75%, 60% and 55%, respectively (Fig. 3). Among the patients suffering from re-occlusions, 8 patients underwent thrombolysis with additional percutaneous interventions in 3 patients. The other 2 patients were treated by surgical interventions (n=1, bypass surgery; n=1, thromboembolectomy). Percutaneous interventions were performed on the 2 limbs suffering from in-stent or in-bypass-stenosis.

Two patients received at least 2 reinterventions during the follow-up period of which one patient who was initially treated by a hybrid approach containing CDT and surgical tromboembolectomy and eventually needed a transtibial leg amputation. The assisted primary patency rates at 3, 6 and 12 months were 100%, 95% and 80%, respectively (Fig. 2).

After 1-year follow-up, a significant improvement was observed in the following dimensions of the SF-36 questionaries: physical function, role physical, vitality, mental health, social functioning and bodily pain (Table 4). However, role emotional showed no statistically significant change over time (p=.09), whereas general health remained unchanged at 6-months but increased over 1-year of follow-up (p=.02). The results of the PCS and its subscales showed a significant decrease after 6-months which stayed statistically significant after 1-year follow-up.

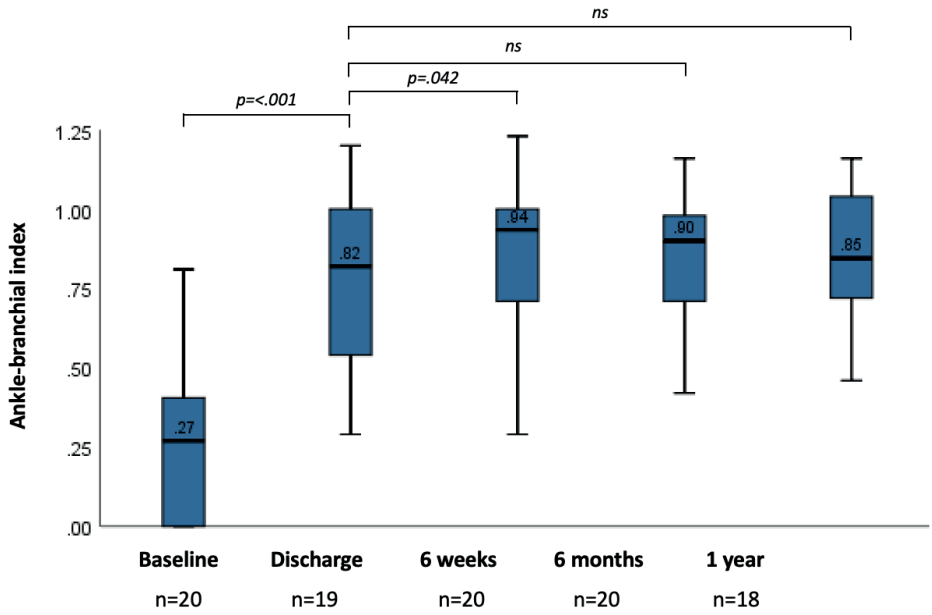

Figure 2. Boxplots of changes in ABI at different timepoints during the trial.

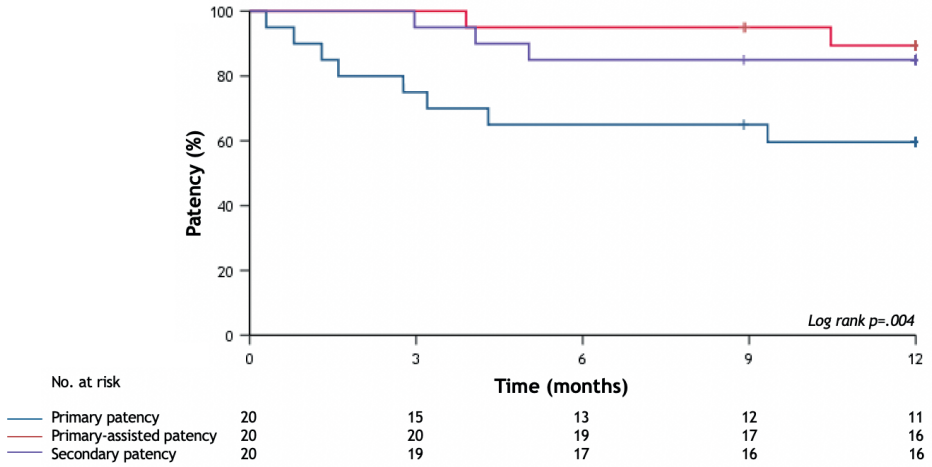

Figure 3. Cumulative Kaplan-Meier estimate of the patency rates following endovascular treatment.

Contrast-enhanced thrombolysis for thromboembolic obstructions in peripheral arteries was performed in 20 patients, of which 10 were treated with UK and 10 with rtPA. Baseline characteristics of the included patients and occlusions characteristics are listed in Table 3.

Primary outcomes

Contrast enhanced thrombolysis was technically feasible in all 20 patients. In terms of the primary safety outcome, no major haemorrhagic complications nor any events of systemic allergic reactions, in-hospital mortality or 30-day mortality related to the microbubbles or ultrasound could be reported.

Table 5. SF36 and PCS on admission, 6-months and 1-year.

SF36	Physical function	Role physical	Role Emotional	Vitality	Mental Health	Social functioning	Bodily pain	General health
Admission	50 (0-100)	.00 (0-100)	100 (0-100)	40 (0-100)	60 (0-100)	50 (0-100)	25 (0-75)	50 (0-100)
6 months	50 (0-100)	100 (0-100)	100 (0-100)	60 (0-100)	80 (40-100)	75 (0-100)	100 (20-100)	50 (0-100)
1 year	50 (0-100)	100 (0-100)	100 (0-100)	60 (20-100)	80 (20-100)	100 (0-100)	100 (0-100)	50 (0-100)
p*	.00	.00	.64	.00	.00	.05	.00	.22
p†	.00	.00	.09	.02	.00	.01	.00	.02

PCS	Total	Rumination	Magnification	Helplessness
Admission	18 (4-47)	8 (0-16)	3 (0-9)	6.5 (0-22)
6 months	7 (0-21)	1.5 (0-9)	0 (0-6)	4 (0-9)
1 year	5 (0-20)	3.5 (0-8)	0 (0-6)	1 (0-6)
p*	.01	.01	.01	.01
p†	.01	.01	.00	.00

Data are presented as mean ± standard deviations (SD).

* *p* value of difference between admission and 6 months.

† *p* value of difference between admission and 12 months.

Secondary outcomes

Secondary outcomes were analysed in 19 patients. One patient was excluded from statistical analysis after receiving microbubbles therapy, as the pre-treatment plan was to use contrast enhanced sonothrombolysis for rapid restoration of blood flow as well as to facilitate an easier access for the surgical procedure of underlying vascular lesions. Due to insufficient file documentation, the analysis of the VAS scores, microcirculation and ABI measurements of another patient could not be performed.

Secondary outcomes regarding treatment characteristics and results are summarized in Table 4. Median time until return to flow at duplex ultrasound was established after 23.1 hours (range 3.1-46.5 hours). In nine patients (50%), return to flow at duplex ultrasound was achieved within 24 hours. Median time until revascularisation at angiography was established within 25.5 hours (range 6.0-81.0 hours). Median duration of total thrombolytic therapy was 47.5 hours (range 6.0-81.0 hours).

Angiographic patency was reached in all cases. In 74% of all cases (n=14) additional percutaneous interventions were required to treat underlying stenotic lesions (e.g., Percutaneous Transluminal Angioplasty (PTA) with or without stent placement). In four cases additional surgical interventions were performed, in one case a femoral-popliteal bypass and in three cases thromboembolectomy. All patients were discharged with a patent treated native artery or bypass graft after additional procedures. After thrombolysis, clinical improvement was observed in 85% (n=17) of all cases. One case in the UK group had mildly worsened in clinical status due to a poor distal run-off which resulted in an early reocclusion of the target artery.

Although no major bleeding complications occurred in any of the groups, minor bleeding at the sheath insertion site or groin hematoma was noted in three cases (16%). None of these patients required volume substitution or surgical evacuation. All cases with minor bleeding complications showed APTT and fibrinogen values within normal ranges, whereas in one patient the INR was slightly elevated 1.25 g/dL on the day of the bleeding complication.

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Discussion

The Microbubbles and UltraSound-accelerated Thrombolysis (MUST) trial is a single-arm phase II trial. This is the first study that shows the safety and technical feasibility of CEST for acute peripheral arterial occlusion of native arteries and bypass grafts.

CDT showed to be an effective treatment in restoring arterial patency and reducing symptoms of acute lower limb ischemia. However, according to the recently published European Society of Cardiology/European Society for Vascular Surgery guideline on peripheral arterial disease, acute lower limb ischemia is still afflicted with a high rate of systemic complications and mortality, even after successful revascularisation(1). Several endovascular techniques have been addressed to enhance the effects of CDT. The first randomized trial comparing endoluminal ultrasound accelerated thrombolysis to standard catheter directed thrombolysis showed a reduction in therapy duration and administered fibrinolytics agents(11). Nevertheless, the ultrasound accelerated thrombolysis did not seem superior to the conventional therapy with respect to the rate of major bleeding complications (11% vs. 6%, respectively).

A potential accelerator of the current thrombolytic therapy is the application of ultrasound with intravenous microbubbles, which has already shown its therapeutic effect in animal studies with large peripheral arterial occlusions(8). There is, however, relatively little data on the use of CEST in patients, mostly limited to small occluded vessels as in myocardial infarction. A recent randomized trial in

ST-segment elevation myocardial infarction comparing low mechanical impulses to high mechanical impulses showed beneficial effects on infarct size with sustained improvements in systolic function at follow-up (12).

The present data demonstrates that this novel treatment is feasible and provides evidence of its safety without any occurrence of side-effects related to the microbubbles, as well as significant improvement in both arterial flow and pain scores. Interestingly, the arterial flow measurements further increased at 6 weeks after the intervention, which may be attributed to successful revascularisation as well as a greater adherence to walking training.

Our results differ from earlier research on the considerably shorter therapy time of 51 hours in the UK-group, while the duration of therapy in the rtPA-group did not differ (UK: 51h (6-81) vs. 67h (4-304), rtPA: 25h (20-73) vs. 20h (2.0-46.0)). The relatively long duration of therapy in the rtPA-group might be explained by the low-dose protocol as well as the long interval between follow-up angiographies. It should be noted that a thorough assessment of the duration of therapy of the rtPA-group is difficult due to the large variation in treatment protocols between previous studies(13).

Lower rates of bleeding complications were observed in this small series of patients when compared to the literature. The overall 1-year amputation and mortality rates in this study were both 2%, considerably lower than the 25% mortality rate and 13% amputation rate reported by Koraen et al(14). Considering the study design and sample size, the findings on adverse events of this study should nevertheless be interpreted with caution.

Up until this day the measurements of fibrinogen depletion during thrombolysis are used to predict bleeding complications or guide in fibrinolytic protocols. Recent studies, however, found no association between a drop in or low level of fibrinogen levels and haemorrhagic complications(1). In this study, the levels of plasma fibrinogen reached levels < 1.0 g/L in two patients without facing any bleeding complications, whereas in the three patients with minor bleedings the fibrinogen levels (>1.5 g/L) were all within normal limits on the day of occurrence.

It has been reported that the severity of ischemia might influence success of thrombolysis as well as mortality and morbidity rates. Korn et al. found a relief of ischemia rate of 43% at 2-years and a combined 30-day mortality and morbidity rate of 18% in the group presenting with acute onset claudication. However, in the group of limb-threatening ischemia a significant higher morbidity and mortality rate was seen (p=.02)(15). This resulted in guidelines advising conservative treatment in patients with viable limbs. In our cohort, only two patients with non-threatened

limbs were successfully treated with thrombolysis and maintained patency during the follow-up. One patient, however, developed a groin haematoma 14 days after discharge, which did not require any surgical intervention. Further research should try to eliminate the appropriate treatment indications of patients with non-threatened limb ischemia.

Shortly after the manufactory difficulties in the production of UK in 2018, we were obliged to alter the thrombolytic agent to rtPA. Even though it has been established that UK and rtPA are equally effective and complication rates do not differ, we still recognize the sample size as a limitation of our study for further analysis of the secondary outcomes. We believe nonetheless that the patient numbers are large enough to provide conclusions regarding the safety of this treatment, which may be of use for the design of future studies.

Since the scope of this study was to demonstrate the safety and feasibility of CEST in a relatively small patient cohort, no global assertions on the efficacy of CEST can be made. To further validate evidence of benefit for CEST it is paramount to perform a large randomized controlled trial with a comparative group. Another promising line of research would be non-invasive thrombolysis with thrombolytic-entrapped microbubbles for targeted thrombolytic delivery(16). Theoretically, this so-called ultrasound-facilitated thrombolysis could increase thrombus dissolution by fibrin targeted microbubble adhesion combined with high-intensity ultrasound leading to a local release of the preloaded thrombolytic agents(17). Whilst some studies provide proof of concept, additional preclinical studies are necessary to further illuminate the clinical potential of accelerate drug delivery.

Conclusions

In conclusion, the findings of this first-in-human trial show promising results regarding its safety and technical feasibility with respect to an experimental contrast-enhanced ultrasound technique in patients with large peripheral arterial occlusions. Contrast enhanced sonothrombolysis has the potential to improve CDT. The results of this study suggest further research on technical innovations focused on the optimization of CDT in order to both increase efficacy and lower risks of haemorrhagic complication to eventually decrease length of in-hospital-stay.

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Conflict of interest

None.

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Chapter 10: Summary, discussion and future perspectives

Summary

Acute limb ischemia is a serious condition where the blood flow to the affected limb is insufficient for its need. The condition is both limb and life threatening and requires urgent intervention. Currently the most commonly used treatment for acute limb ischemia is catheter directed intra-arterial thrombolysis. It is a minimally invasive therapy, but it has the drawback of being time consuming and it carries a risk of (major) bleeding. This thesis focuses on contrast enhanced sonothrombolysis (CEST) for acute peripheral arterial thrombosis. CEST is a form of thrombolysis where ultrasound contrast agents (a.k.a. microbubbles) and ultrasound are used to enhance thrombolysis.

Part I of this thesis is focused on results of current clinical practice and on what we know about CEST for peripheral arterial thrombosis. In **chapter 2**, we retrospectively reviewed the results of 2 thrombolytic protocols (low- vs high-dose urokinase) administered on a regular surgical ward. Although we found no difference in technical success rate or 6 months amputation-free survival, there was a significantly lower rate of major bleeding complications in the low-dose group. A systematic review of all *in vivo* studies on contrast-enhanced sonothrombolysis in a setting of arterial thrombosis is given in **chapter 3**. A total of 26 preclinical and 9 clinical trials were included in the review, most of these were done in a setting of ischemic stroke or myocardial infarction. Due to high heterogeneity it was not possible to perform a meta-analysis, but almost all studies showed a higher recanalization rate of the affected artery in the group that received a form of CEST.

Part II of this thesis focuses on the results of our preclinical trials. Based on several studies that investigated the benefit of CEST in a setting of ischemic stroke or myocardial infarction, we developed a porcine model for investigating these in acute peripheral arterial occlusion. In **chapter 4** we present this model and how to prevent possible allergic reactions of the pigs to the ultrasound contrast agents. This model is implemented in **chapters 5**, **6** and **7**, where we used it to investigate different aspects of microbubbles and with it different protocols for CEST . In **chapter 5** we investigated the effect of intravenous microbubbles and local ultrasound added to standard intra-arterial catheter directed thrombolysis (sCDT) and compared this with sCDT. We found a higher arterial blood flow and a significant lower thrombus weight at the end of the procedure in the CEST group compared to the sCDT group. In **chapter 6**, we explored the possibility of using microbubbles as a carrier for the thrombolytic agent. Microbubbles, loaded with Urokinase and modified to adhere to thrombus, were administered intravenously and destroyed with high mechanical index ultrasound at the site of the thrombus. As control group we administered intravenous Urokinase alone, combined with the same regiment of ultrasound. We found a significantly lower thrombus weight

in the CEST group at the end of the experiment and 2 out of 5 pigs in the CEST group had a complete or nearly complete return to the baseline arterial blood flow in the affected artery compared to none in the control group. In **chapter 7** we evaluate the feasibility of administering microbubbles intra-arterially via an ultrasound-emitting catheter. In an *in vitro* set-up we tested if the destruction of the microbubbles, in turn leading to destruction of thrombus, could be achieved by the ultrasound-emitting catheter and whether there was a difference when the microbubbles were administered directly through the catheter versus via the blood stream. We found that microbubbles did get destroyed by the ultrasound of the catheter and that this was not influenced by the mode of administration of the microbubbles. We tested the lytic effect of this combination in an *in vivo* model and found a significant lower thrombus weight in this group when compared to our historical controls.

In part III of this thesis we present the protocol for the first clinical trial testing the safety and feasibility of CEST in acute peripheral arterial occlusion (**chapter 8**) and the results of this trial (**chapter 9**). A total of 20 patients were treated with CEST on a regular surgical ward. We found CEST to be feasible in a clinical setting and there were no serious adverse events related to the experimental treatment.

Discussion and future perspectives

In this thesis we investigated several protocols of CEST in order to improve standard intra-arterial thrombolytic therapy. Standard catheter-directed intra-arterial thrombolysis has the benefit of being minimally invasive. Patients don't require general anesthetics and the thrombolytic agent reaches small arteries that cannot be reached via surgical thrombectomy, which helps to improve outflow in the affected extremity. Drawbacks of sCDT are the intrinsic risk of bleeding that comes with the use of a fibrinolytic agent and the time it takes for the thrombus to dissolve, i.e. the time it takes to restore direct blood flow to the extremity. Although the treatment is minimally invasive, it still requires placement of an intra-arterial catheter. Patients have to stay in a supine position during treatment to prevent dislodgement of the catheter, which adds to the burden of this form of therapy. In the next paragraphs we will discuss possible improvements on sCDT for peripheral arterial occlusion as investigated in this thesis.

Minimalizing risk of major bleeding

Thrombolytic therapy carries a risk of major bleeding, the most important of which is intracerebral hemorrhage. Thrombolytic protocols vary widely in the literature: there are different thrombolytic agents, different modes of delivery, different dosages, and different durations of therapy. In our study in a retrospective cohort in **chapter 2**, we found that a protocol with low dose Urokinase gave the same technical success rate as a higher dose protocol, but a significantly lower

incidence of major bleeding. Duration of therapy however, did increase with a lower administered dosage of the fibrinolytic agent. These findings concur with a recent systematic review by Ebben et al.[1] They found a longer mean duration of therapy (32.7 hours vs 21.9 hours) when low-dose protocols were compared with high-dose protocols. In high-dose protocols the pooled mean rate for major bleeding was 8.9% versus 6.3% for low-dose protocols. Technical success rates were comparable, with pooled mean rates of 74.9% versus 74.1% for high-dose versus low-dose protocols. Another interesting finding in this review was a higher overall bleeding risk for rt-PA (19.2%) and tPA (18.6%) versus Urokinase (15.1%). The authors of this review conclude there is a lack of comparative prospective data and heterogeneity of studies made it impossible to perform a meta-analysis. For future research it would be interesting to prospectively compare different thrombolytic protocols for the most well-known fibrinolytic agents with regards to effectiveness and risk of complications.

In **chapter 3** we give an overview of all in vivo studies on CEST in arterial thrombosis. CEST is a form of thrombolysis where a combination of microbubbles and ultrasound is added to the standard thrombolytic regiment. Microbubbles are ultrasound contrast agents that have been widely used to enhance duplex imaging. They have been shown to be safe as a diagnostic tool and are now under investigation for their possible therapeutic properties. They can be influenced by ultrasound to either oscillate or cavitate. This causes microstreaming of fluid and with it shear stress to the surface of the clot, resulting in a larger clot surface for the fibrinolytic agent to interact with. In our systematic review we found 26 preclinical and 9 clinical trials on CEST for arterial thrombosis. Of the 26 preclinical trials only 1 reported evidence of focal hemorrhage in the cerebral infarct area, but this occurred in all treatment arms. The main bleeding complication in the 9 clinical trials on CEST was intracranial hemorrhage. Of these trials, 8 were conducted in ischemic stroke patients. Overall there were no significant differences in occurrence of intracranial hemorrhage between the treatment arms, with the exception of the TUCSON trial by Molina et al., published in 2009[2]. This was a randomized double blind dose-escalation study using CEST for patients with ischemic stroke. It was terminated prematurely due to a higher incidence of intracranial hemorrhage in the second tier of the study. The investigators found other likely contributing factors that might explain the higher incidence of intracerebral hemorrhage in this group, namely a larger infarct area pretreatment and a higher systolic blood pressure. The termination of this study had a huge impact on further clinical trials, even though the trial was underpowered and there were reasonable explanations for a higher rate of intracerebral hemorrhage in the second cohort. We feel this trial shouldn't stop research on CEST for patients with peripheral arterial occlusion, as there are some important differences with patients suffering from ischemic stroke and there is a lot to gain. Cerebral ischemia carries an intrinsic risk of hemorrhagic transformation

with incidences in literature that vary between 15 and 43%[3]. CEST for patients with stroke in these studies invariably contained an intravenous administered fibrinolytic agent. In sCDT, the fibrinolytic agent is administered intra-arterially, as intravenous administration has been proven to give a higher incidence of major bleeding complications[4]. As the occluded artery in ischemic stroke is a cerebral artery, ultrasound directed at the site of occlusion might have a direct effect on surrounding brain tissue. These aspects are very different for CEST in peripheral arterial occlusion, since the lesion typically lies a considerable distance from the cerebrum. The clinical trial conducted in patients with myocardial infarction did not report any intracerebral hemorrhaging[5]. In our trial testing safety and feasibility of CEST in 20 patients with acute peripheral arterial occlusion presented in **chapter 9**, we did not find any major bleeding complications.

Shortening time to reperfusion

In **chapter 2** we found a longer duration of therapy was needed when a lower dose of thrombolytic agent was used. The low-dose protocol gave a lower risk of major bleeding, but at the cost of a longer time to reperfusion. A short time to reperfusion is essential in keeping tissue loss to an absolute minimum. The longer the duration of ischemia, the higher both amputation and mortality rate[6]. A longer duration of therapy prolongs the burden for the patient and will also add to a higher radiation exposure due to more control angiographies. The overall risk of complications also increases with the duration of therapy[7]. Furthermore a longer duration of therapy might add to costs due to a longer hospitalization time. In **chapter 5** we investigate CEST as a way to improve time to reperfusion. In our short duration experiments (3 hours of therapy) we tested the effect of CEST vs sCDT. Even in this short amount of time we found significant reperfusion in several of the animals in the CEST group versus none in the control group. Thrombus weights were significantly higher in the control group versus the CEST group. The addition of ultrasound to CDT has already been implemented in clinical practice by ways of an ultrasound emitting catheter. In 2007 Motarjeme reported on his clinical experience with this catheter using it for ultrasound enhanced thrombolysis in both arterial and venous occlusions[8]. He found a shorter time to reach complete clot lysis than historically reported data. These findings were later supported by a randomized trial in 2015 comparing sCDT with CDT using an ultrasound emitting catheter[9]. As this ultrasound emitting catheter seems to reduce time to reperfusion, we tested the effect of this catheter on microbubbles *in vitro* and what this form of CEST might result to *in vivo* in **chapter 7**. We found that the ultrasound emitting catheter does destroy the microbubbles, aiding in further erosion of the clot. This finding might help for future clinical studies, as this mode of delivering ultrasound to the site of the occlusion is already in use in daily practice. Another finding of the study in chapter 7 is a significantly lower mean thrombus weight compared to our previous control group. Both the study in chapter 5 and chapter 7 suggest that

adding microbubbles and ultrasound to sCDT hastens dissolution of the thrombus and as such will shorten time to reperfusion.

Little has been reported on CEST in a setting of peripheral arterial thrombosis, but there have been some *in vivo* studies that support our findings in a setting of ischemic stroke or myocardial infarction. An overview of these studies is given in **chapter 3**. As was the case with the systematic review on CDT protocols, there is a high heterogeneity amongst studies and therefore it was impossible to perform a meta-analysis. That said, almost all studies included in this review showed greater rates of recanalization of the previously occluded artery in a group where CEST was used compared to any other treatment. The two studies that did not show greater rate of recanalization, showed similar rates to the best compared group. As mentioned before, the TUCSON trial was terminated prematurely due to a higher incidence of intracranial hemorrhage in the second tier of the study[2]. It was therefore underpowered, but there was a trend towards a higher recanalization rate in CEST group as well as a higher rate of functional independence at 3 months.

Minimalizing burden for the patient

sCDT demands full cooperation from the patient. The intra-arterial catheter is placed percutaneously and kept in place during treatment. In order to prevent dislodgement of the catheter and with it major bleeding complication and/or failed therapy, the patient has to stay in a supine position. As treatment can take up to 3 days, the patient has to stay in bed and is completely dependent on care during this period. Reducing time to reperfusion, and with it therapy duration, would reduce the time that a patient is bedridden.

Another possible way to reduce burden for the patient is explored in **chapter 6**, where we investigate not only the thrombolytic properties of microbubbles, but also their carrier function. In this study we loaded the microbubbles with Urokinase. The Urokinase is encapsulated in the microbubble and released locally under influence of Ultrasound. While the Urokinase is loaded in the microbubble it is less biologically active, theoretically resulting in a lower risk of bleeding complications[10]. We added RGDS, a targeting ligand for thrombus, to the surface of the microbubble so the microbubbles would accumulate at the site of the occlusion. As the targeted Urokinase loaded microbubbles were administered intravenously, we compared them with intravenously administered Urokinase without microbubbles and exposed both groups to high-intensity ultrasound. At the end of the experiment thrombus weight was significantly lower in the CEST group and 2 out of 5 pigs in this group had a (near) complete return of baseline arterial blood flow through the previously occluded artery compared to none in the Urokinase and ultrasound group. We did not find any adverse events, specifically no signs of (intra-cranial) bleeding. Guan et al. recently conducted a similar study

in 43 rabbits where different intravenous CEST protocols were applied during 120 minutes of treatment[11]. They found that targeted microbubbles combined with Urokinase were more effective in achieving recanalization than non-targeted microbubbles. This might be explained by a higher concentration of microbubbles at the site of the occlusion due to the adherence of the microbubbles to thrombus. As these were only short duration experiments in a relatively small amount of subjects, this form of therapy needs further investigation on both safety and efficacy before it can be applied in a clinical setting.

Other future perspectives

In **chapter 9** we present the results of a clinical study investigating CEST in the setting of acute peripheral arterial thrombosis. Although the trial was done in a small number of patients, results are promising: CEST for peripheral arterial thrombosis was feasible in a clinical setting and there were no serious adverse events related to the treatment[12]. It is our intention to conduct a randomized multicenter trial to compare effectivity of CEST vs sCDT in the hope of improving outcomes for patients with acute peripheral arterial occlusion. Outcomes for this trial will include time to reperfusion, limb salvage, overall survival, quality of life parameters and long term results.

There is some evidence of beneficial effect of microbubbles on microcirculation and with this on outflow[13,14]. As the 'no-reflow' phenomenon is very likely the result of loss of outflow, this offers some interesting possibilities for future investigations.

Another aspect of microbubbles that can be further explored is their carrier capacity. As mentioned previously we can load medication in the microsphere, making it biologically inactive until the microbubble is destroyed at the intended site. Adding a targeting ligand to the bubble makes it adhere to the area of interest, in our case thrombus surface. A recent development in the area of therapeutic use of microbubbles is the magnetic microbubble[15]. This new form of microbubble can be magnetically directed to a focus area. Another interesting recent development in the area of CEST, is the use of nanodroplets instead of microbubbles. The smaller size of nanodroplets, 100-300nm, allows them to penetrate deeper into a clot. Nanodroplets are also more stable than microbubbles, with a longer half-life in the circulation. Both the size and the stability of nanodroplets might make them even more effective in disrupting a thrombus[16]. With all these current developments, local thrombolysis via a simple intravenous injection might very well be possible in the near future.

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Chapter 11: Dutch summary – Nederlandse samenvatting

Een acuut tekort aan bloed in een ledemaat ontstaat wanneer de slagader (arterie) naar dat ledemaat plotseling afgesloten wordt door bijvoorbeeld een stolsel (trombus). Een dergelijke acute arteriële afsluiting (occlusie) vormt een gevaar voor zowel het aangedane ledemaat als voor het leven en dient zo spoedig mogelijk verholpen te worden. De meest gangbare vorm van behandeling heden ten dage is trombolyse behandeling via een intra-arteriële katheter. Hierbij wordt de arterie aangeprikt en wordt een katheter in het vat opgevoerd naar de plek waar de afsluiting zich bevindt. Via de katheter wordt continue een sterk stolsel oplossend middel (trombolyticum, bijv. Urokinase of Alteplase) toegediend ter plaatse van de trombus. Deze vorm van behandeling is minimaal invasief, maar heeft als belangrijke nadelen dat het tijd kost en dat er een risico is op ernstige bloedingen, vooral in het hoofd. Vanuit de neurologie en cardiologie kwam een nieuwe techniek om trombusmassa sneller op te lossen (lyseren) en mogelijk zo uitkomsten voor de patiënt te verbeteren. Bij deze techniek werden echocontrastmiddelen, ook wel microbubbels of microbellen genoemd, gebruikt in combinatie met echo (ultrageluid) en een trombolyticum. De microbellen trillen en barsten onder invloed van ultrageluid, wat resulteert in mechanische disruptie van de trombus. Hierdoor kunnen stolsels sneller opgelost worden. We noemen dit contrast-versterkte sonotrombolyse (CVST). Dit proefschrift richt zich op verschillende aspecten van CVST voor patiënten met een acute arteriële afsluiting, met als uiteindelijk doel de uitkomst voor deze patiënten te verbeteren.

Het proefschrift is opgebouwd in 3 delen. In deel I richten we ons op de huidige situatie in de kliniek en geven we een overzicht van wat er bekend is over CVST bij perifere arteriële trombose. In **hoofdstuk 2** bekijken we retrospectief de resultaten van 2 trombolyseprotocollen: lage versus hoge dosis Urokinase. Er bleek geen verschil in technisch succes of amputatie vrije overleving na 6 maanden tussen de beide groepen, maar er werden wel significant minder ernstige bloedingen gevonden in de groep die een lage dosis Urokinase toegediend kreeg. In **hoofdstuk** 3 presenteren we een systematische review van alle in vivo studies naar het gebruik van CVST in arteriële afsluitingen. In totaal konden we 26 preklinische en 9 klinische studies includeren in de review. Door de hoge heterogeniteit van de studies was het niet mogelijk om een meta-analyse te verrichten, maar vrijwel alle studies toonden een hoger rekanalisatiepercentage in de groep die een vorm van CVST kreeg.

Deel II van dit proefschrift richt zich op de resultaten van preklinische studies naar CVST. In **hoofdstuk 4** presenteren we een varkensmodel voor het onderzoeken van deze nieuwe vorm van trombolyse bij acute perifere arteriële trombose en stellen we een protocol voor ter voorkoming van allergische reacties op de microbellen bij de proefdieren. Bovengenoemd diermodel implementeren we in **hoofdstuk 5, 6 en 7**, waarbij verschillende therapeutische mogelijkheden van de microbellen worden onderzocht. In **hoofdstuk 5** vergelijken we via het infuus (intraveneus)

toegediende microbellen gecombineerd met lokaal ultrageluid en standaard intra-arteriële trombolyse met standaard intra-arteriële trombolyse alleen. We vonden een hogere arteriële stroomsnelheid en een significant lager trombusgewicht in de groep met de CVST aan het eind van het experiment. In **hoofdstuk 6** onderzoeken we de mogelijkheid om microbellen als een vervoermiddel voor het trombolyticum te gebruiken. Urokinase werd in de microbellen geladen en de bellen werden zo geprepareerd dat ze zich zouden binden aan trombusmateriaal. De microbellen werden intraveneus toegediend en ter plaatse van de afsluiting met ultrageluid kapot gemaakt. De zo behandelde groep werd vergeleken met een groep die alleen Urokinase intraveneus kreeg plus ultrageluid. De groep die microbellen toegediend kreeg had een significant lager trombusgewicht aan het eind van het experiment en 2 van de 5 varkens in deze groep hadden een (vrijwel) complete terugkeer van doorstroming in de aangedane arterie. Dit in tegenstelling tot de controle groep waar dit in geen van de experimenten het geval was. In **hoofdstuk 7** onderzoeken we de mogelijkheid om de microbellen intra-arterieel toe te dienen via een ultrageluid katheter. In-vitro testten we of de microbellen door de ultrageluid katheter kapot gemaakt werden en zo ja, of het uitmaakt op welke wijze ze toegediend werden: via de katheter of via het lumen van het vat. We zagen dat de microbellen door de katheter kapot gemaakt werden en dat dit niet beïnvloed werd door de wijze van toedienen. We testten het lytische effect van deze wijze van toedienen van zowel microbellen als ultrageluid in een proefdiersetting en vonden ook hier een significant lager trombusgewicht aan het eind van het experiment vergeleken met onze eerdere controle groep met standaard intra-arteriële trombolyse.

In het derde deel van dit proefschrift presenteren we de eerste klinische studie naar CVST bij mensen. Aan de hand van de resultaten van de studies uit hoofdstuk 5, 6 en 7 schreven we een protocol voor een studie naar de veiligheid en haalbaarheid van CVST bij mensen (**hoofdstuk 8**). In **hoofdstuk 9** presenteren we de resultaten van deze studie. In totaal werden 20 patiënten behandeld met CVST. De behandeling bleek haalbaar in een klinische setting en er waren geen ernstige complicaties gerelateerd aan de experimentele behandeling.

Concluderend lijkt CVST een gunstig effect te hebben op duur en effectiviteit van trombolysebehandeling van acute arteriële trombose en lijkt het ook een haalbare, veilige therapie te zijn in een klinische setting. Met CVST kunnen lagere doses van het trombolyticum geven, ten einde bloedingscomplicaties te verminderen. Daarnaast lijkt het mogelijk een snellere trombolyse te verkrijgen zonder patiënten bloot te stellen aan additieve risico's. Een gerandomiseerd trial om dit te onderzoeken is momenteel in voorbereiding. Een sneller herstel van bloedtoevoer naar het bedreigde ledemaat zal de belasting voor de patiënt verminderen en hopelijk het uiteindelijke resultaat verbeteren. Er is echter nog veel onderzoek nodig voor we dit daadwerkelijk in een dagelijkse klinische praktijk kunnen implementeren.

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The Road Not Taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.

Robert Frost, 1916

Thank you - Dankwoord

Een proefschrift is het product van een vaak lange reis. Eén die je niet alleen aflegt. Bij de eerste stappen wordt je koers vaak voor je bepaald. Iemand pakt je hand en zet je op het pad: “Kijk, daar is je doel”. En inderdaad, daar is het. Helder zichtbaar, goed afgetekend tegen de horizon, je kan het bijna aanraken. Maar de tijd en het leven oefenen onherroepelijk hun invloed uit. Het doel flikkert, je twijfelt. Fata Morgana? Het pad kronkelt en slingert, vertakt. Wordt beïnvloed door bagage, wandelstok, weer, gezelschap. En dan ben je waar ooit je doel lag en weet je: je moet nog een stukje verder. Misschien maar goed ook. Voor de mensen die me gezelschap hielden en houden op deze reis: jullie hebben de reis verlicht, het avontuur vergroot en me gebracht tot waar ik nu ben. En we zijn er nog niet, reis je nog wat langer mee?

[TL;DR] (Voor de mensen die het dankwoord wat lang vinden): dank je wel en sorry als ik je vergeten ben!

In 2019 zat ik in de aula, terwijl Harm Ebben - met wie ik meerdere van mijn onderzoeken verrichtte - zijn proefschrift verdedigde. Na de verdediging en het verkrijgen van de graad van doctor, richtte zijn promotor, professor Willem Wisselink, het woord tot hem: ‘Beste Harm, het valt me op dat je in je dankwoord een essentiële groep bent vergeten!’ Harms blik hield ergens het midden tussen beduusdheid en ongerustheid. Op wie kon de professor doelen? ‘Je besteedt geen enkele aandacht aan de roze wezentjes die jouw onderzoek mogelijk hebben gemaakt!’ Ik wist meteen, deze vergissing zal ik niet begaan. Dus Willem, slechts een tweede plek voor jou in dit dankwoord. Want die roze viervoeters, die waren er ook voor mij.

Mooie, warme, roze dames, onze **pink ladies**. Jullie hebben al slapend dit onderzoek en alles wat daar nog uit zal voortkomen mogelijk gemaakt. Na een klein prikje werden jullie suf en kregen jullie gelukkig niets meer mee van alles. En toch. ‘I dub thee spiderpig’, schreef een student in hanepoterige letters op 1 van onze onderzoekspapieren. Ook ik zag persoonlijkheid en karakter in jullie, nieuwsgierige knorders. Het voelt gek om iemand te bedanken die geen keuze had. Toch ben ik dankbaar dat jullie er waren. Ik hoop dat we de kennis waar jullie aan hebben bijgedragen binnenkort kunnen toepassen in de kliniek en daarmee het leed voor onze patiënten zullen verminderen. En voor jullie wens ik veel modderpoeltjes in knorretjeshemel.

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Pauli

….
Jungske leef, nôw luuster,
zag ik in d'n duuster,
de kins mich dao aan halde wat ik nôw verklaor:
as mich is gegaeve
dén te meuge laeve,
zal ik nog van dich halde euver vieftig jaor.
..*

Het zijn 'golde jaore' en ik ben intens dankbaar dat jij er bent. Jij staat in het centrum van alles, alle paden leiden naar jou: kompas, tegenwicht, steunpilaar, warmte, geluk. Wat hebben we veel meegemaakt samen en wat hoop ik dat we nog heel lang heel veel mee zullen maken samen. We worden 115 en sterven tegelijk doordat er een koe op onze hoofden valt. (De koe loopt natuurlijk ongedeerd weg.) Hald mich nog maar efkes vas en luets mich pas as ik alles vergaete bin los, dan doon ik dat auk mit dich.

** Wengske aan wengske, De Vrijbuiters, 1980*

...

Straks aan d'n euverkant van de nach dao wach de veurjaorszôn.
Achter de nach dao wach op os de zon.

Frans Pollux, 2013